

Host factors and compartments accessed by *Salmonella*

Typhimurium for intracellular growth and survival

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Abstract

Salmonella serovars are facultative, intracellular gastrointestinal pathogens responsible for causing diseases ranging from diarrhea and enterocolitis to severe, systemic fatal infections in humans and animals, and represent a major public health and economic burden worldwide. Within infected host cells, *Salmonella* resides within a membrane-bound compartment called the *Salmonella*-containing vacuole (SCV). Formation of the SCV has been reported both in epithelial and macrophage cells. The SCV has been regarded as a nutrient deprived compartment because of its isolation from both extra- and intracellular sources of nutrients. However, despite apparent nutrient limitation within the SCV, *Salmonella* is still able to replicate in the SCV, indicating adaptation to this intracellular environment.

Despite rapid progress in understanding the molecular functions of virulence factors of *Salmonella* involved in intracellular survival and replication, a fundamental question regarding the source(s) of nutrients for growth of *Salmonella* within the SCV is still not completely understood. Our study shows that *Salmonella* Typhimurium acquires small peptides by co-opting the host cell cytosolic protein turnover pathway known as chaperone-mediated autophagy (CMA). CMA is a selective host cell protein turnover pathway active in all cell types and is involved in the transport of cytosolic proteins into lysosomes for degradation. An estimated 30% of all cytosolic proteins are turned over through this mechanism. Here we show for both intracellular *Salmonella* and in purified SCVs that the SCV is associated with the key components of the CMA, LAMP-2A and Hsc73 and upon inhibiting CMA either by chemical inhibitors or by knocking down LAMP-2A in cells affects the intracellular growth of peptide-dependent mutants of *Salmonella*. Furthermore, the SCV selectively associated with CMA components and excludes association with liposomal markers suggesting no interaction with the lysosomes. Furthermore, for our studies we have also developed a novel method for isolating intact SCV using paramagnetic nanoparticles.

The results of this study highlight a unique phenomenon in host-pathogen interaction wherein an intracellular pathogen monitors the fitness of their infected host cells and are able to couple their own intracellular proliferation rate to the health status of the host.

Zusammenfassung

Verschiedene Serovare der Spezies *Salmonella* sind fakultativ intrazelluläre gastrointestinale Pathogene. Sie sind ursächlich für eine Reihe von Erkrankungen wie Salmonellose, Diarrhö, Enterokolitis sowie schwerwiegenden systemischen Infektionen bei Mensch und Tier. *Salmonella* stellt daher ein bedeutendes Public Health Problem dar und verursacht weltweit einen hohen ökonomischen Schaden.

Ähnlich wie bei anderen intrazellulären Pathogenen befindet sich *Salmonella* innerhalb von infizierten Wirtszellen in einem durch Membranen abgetrennten Kompartiment, der sogenannten “*Salmonella*-Containing Vacuole (SCV)“. Das Vorhandensein dieser SCV wurde sowohl in Epithel- als auch in Makrophagen-Zellen beobachtet.

Da das Innere dieser SCV sowohl von intrazellulären als auch von extrazellulären Nährstoffquellen abgeschnitten ist, geht man von einem Nährstoffmangel in diesem Kompartiment aus. Trotz dieses offensichtlich vorliegenden Nährstoffmangels bleibt *Salmonella* aber in der Lage sich innerhalb der SCV zu replizieren. Dies spricht für eine erfolgreiche Adaptation an die Bedingungen dieses intrazellulären Milieus.

Trotz des umfangreichen Wissens über den intrazellulären Status von *Salmonella* und den dabei involvierten Virulenzfaktoren sowie deren molekularen Funktionen, ist die Art der Nährstoffquelle welche *Salmonella* innerhalb der SCV nutzt bisher noch nicht völlig geklärt. In unserer Studie konnten wir allerdings zeigen das *Salmonella* Typhimurium mit dem wirtseigenen „Cell Chaperone mediated Autophagy (CMA)“-abhängigen zytosolischen Protein-Metabolismus interagiert. Bei CMA handelt es sich um einen wirtszelleigenen selektiven Protein-Stoffwechselweg der in allen Zelltypen vorkommt. Er dient dem Transport von zytosolischen Proteinen in die Lysosomen wo sie dann abgebaut werden.

So werden ca. 30% des zytosolischen Proteins über diesen Mechanismus metabolisiert. In unseren Arbeiten konnten wir sowohl für intrazelluläre *Salmonella* als auch für aufgereinigte

SCVs zeigen das diese mit Kernkomponenten des CMA-Systems wie LAMP-2A und Hsc73 assoziiert sind.

Des weiteren konnten wir zeigen das CMA entweder direkt über chemische Inhibitoren oder den Knock Down von LAMP-2A in den Zellen beeinflusst werden konnte was das intrazelluläre Wachstum von peptid-abhängigen *Salmonella* Mutanten beeinflusste. Weiterhin waren die SCVs ausschließlich mit CMA Komponenten und nicht mit liposomalen Markern assoziiert, was gegen eine Interaktion mit den Lysosomen spricht. Zusätzlich wurde eine neue Methode zur Isolation intakter SCV mittels paramagnetischer Nanopartikel entwickelt.

Die Ergebnisse dieser Studie zeigen ein einzigartiges Phänomen auf dem Gebiet der Wirt-Pathogen- Wechselwirkung bei dem ein intrazelluläres Pathogen die Fitness der infizierten Wirtszelle überwacht und in der Lage ist seine eigene intrazelluläre Proliferationsrate an den Gesundheitsstatus der Wirtszelle zu knüpfen.

Abbreviations

APS	Ammonium peroxodisulfate
bp	Base pairs
BCA	2,2' Bicinchoninic acid
BSA	Bovine serum albumin (Fraction V)
C	Celsius
Cat	Chloramphenicol acetyl transferase
cDNA	Complementary DNA
cfu	Colony forming units
Cu	Copper
DCE	Anhydrous 1,2-dichloroethane
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxyribonucleoside triphosphates
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediamino tetraacetic acid
EEA1	Early endosomal antigen 1
EGTA	Ethylene-glycol-bis (2-aminoethyl) tetraacetic acid
FCS	Fetal Calf Serum
Fig.	Figure
GFP	Green Fluorescent protein
h	Hours
IPTG	Isopropyl-thio- β -D-galactopyranoside

kb	Kilo base
Km	Kanamycin
LB	Lennox broth
lgps	lysosomal glycoproteins
LPS	Lipopolysaccharide
LTTR	LysR-type transcriptional regulator
m	Milli
M	Molar
μ	Micro
OD	Optical density
ONPG	o-Nitrophenyl-β-D-galactopyranoside
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Pathogenicity island
p.i.	post-infection
PMSF	Phenylmethanesulphonyl fluoride
ppGpp	Guanosine tetraphosphate
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse-Transcriptase Polymerase Chain Reaction
SCV	<i>Salmonella</i> -containing vacuole
SDS	Sodium dodecylsulfate

SIF	<i>Salmonella</i> -induced filament
SPI	<i>Salmonella</i> pathogenicity island
TBE	Tris-borate-EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tc	Tetracycline
TRIS	2-Amino-2-hydroxymethyl-1,3-propandiol
T3SS	Type III secretion system
X-Gal	5-Bromo-4-chloro-3-indoyl- β -D-galactoside

Introduction

Salmonellosis

Salmonella enterica is a Gram-negative intracellular bacteria belonging to the *Enterobacteriaceae* and which is responsible for gastrointestinal diseases upon ingestion of contaminated food and water. *Salmonella* is a pathogen is of high clinical relevance in both developing and developed nations causing food-borne illness and other diarrheal diseases as well as severe systemic infections and economic losses. Many serovars of *Salmonella* can infect and colonize a wide variety of hosts, with outcomes ranging from sub-clinical infections to life threatening systemic fatal disease (Jones *et al.*, 2008a; Lahiri *et al.*, 2010). *Salmonella* has been successfully isolated both from warm blooded and cold blooded animals (Fookes *et al.*, 2011; Schikora *et al.*, 2011), furthermore, several sub-species of *Salmonella* are known to infect plants. Hence *Salmonella* is often considered as a ‘universal pathogen’ (Fedorka *et al.*, 2000).

Salmonella as pathogen possesses a very high zoonotic potential with millions of fresh human cases reported every year. According to a report by Centre for Disease Control, (CDC, 2013) in the US alone, approximately, 40,000 cases of *Salmonella* are reported each year. Furthermore, of the total report cases, the severity of infection predominates in children, the immunocompromised individuals and the old aged, leading to death of nearly 400 persons every year as a result of acute Salmonellosis. In the European Union, infections due to *Salmonella* are the second most reported bacterial diseases in humans after *Campylobacter* infections (EFSA and ECDC, 2013 Summary report on Zoonosis). In the developing and third world countries, *Salmonella* serovars are responsible for numerous outbreaks within a year thus signifying the frequency with which these serovars persist and cause infections in the environment (Kariuki *et al.*, 2006; Kozak *et al.*, 2013). Both in United States and European Union, reported human cases of salmonellosis are frequently related to *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis (CDC, 2011-National *Salmonella*

Surveillance Annual Data Summary 2009; EFSA and ECDC, 2013-Summary report on Zoonosis). *Salmonella* serovar Enteritidis cases are principally related to contaminated eggs and poultry meat. However, *Salmonella* serovar Typhimurium infections are also the result of consumption of contaminated meat products from pigs, bovine and poultry. Thus, *Salmonella* infections or Salmonellosis remains a major health burden and represents a significant health-related and economic cost to society.

The genus *Salmonella*

The genus *Salmonella* was discovered by an American veterinary pathologist, Daniel Elmer Salmon, in 1885. The genus *Salmonella* refers to facultative, anaerobic intracellular bacteria which exhibit predominant peritrichous motility. *Salmonella* are Gram-negative, rod shaped, non-spore forming bacteria, with diameter ranging from 0.7 to 1.5µm and a length of 2 to 5µm belonging to *Enterobacteriaceae* family (Murray *et al.*, 1999; Coburn *et al.*, 2007). The genus *Salmonella* is closely related to *Escherichia*, however horizontal gene transfer has played an instrumental role in its divergence from the *E. coli* lineage (Baümeler *et al.*, 1998; Retchless and Lawrence, 2010) through acquisition of virulence determinants, pathogenicity islands and plasmids favouring *Salmonella* infection and adaptation to a wide range of hosts. Notably, horizontal gene transfer is responsible for acquisition of *Salmonella*-Pathogenicity island-1 (SPI-1), encoding virulence genes and factors, interestingly, it is absent in *E. Coli* and other non pathogenic Gram negative bacteria (Collazo and Galan, 1997). The SPI-1 encoded gene and virulence proteins are responsible for the invasion of *Salmonella* within host cells (Miller *et al.*, 1995; Hensel 2004).

Classification

The WHO collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris France, maintains and updates the classification of the genus *Salmonella* based on the Kauffmann-White scheme of serotyping (Grimont and Weill, 2007). The genus *Salmonella* consists of two species, *Salmonella enterica* and *Salmonella bongori*. The species

S. enterica is further divided into six sub-species namely, *S. enterica* subsp.*enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV) and, *S. enterica* subsp. *indica* (VI) as shown in Figure 1. The species *S. enterica* has acquired a second pathogenicity island, SPI-2, responsible for establishing a replicative niche for the pathogen within its host (Retchless and Lawrence, 2010). *S. bongori* subsp. (V), as well as subspecies II, IIIa, IIIb, IV and VI are principally isolated from cold-blooded vertebrates and the environment. The *S. enterica* subsp. *enterica* (I), is mainly responsible for causing infections and diseases in humans and other domestic animals (Fierer and Guiney, 2001; Lan *et al.*, 2005).

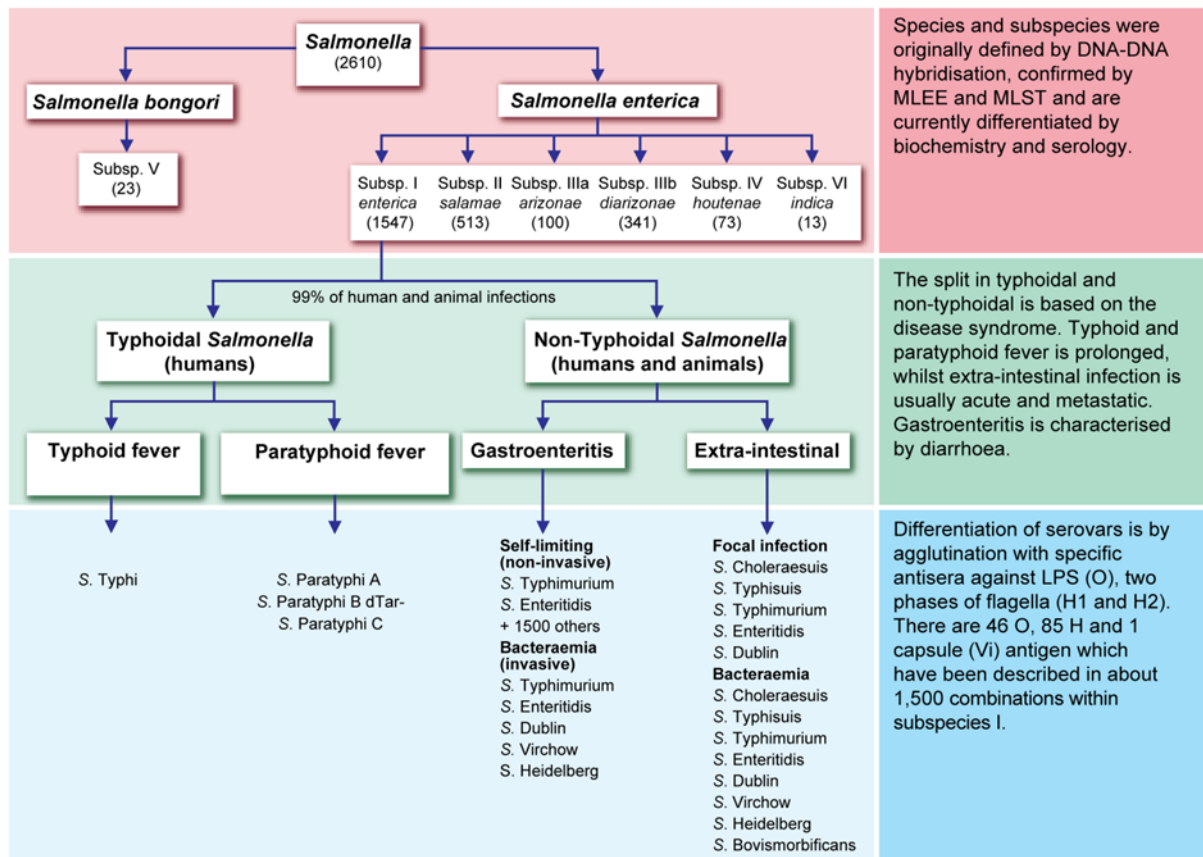


Figure 1. Overview of the current classification of *Salmonella enterica*. The figure represents classification of the genus *Salmonella*, which is divided into two species *Salmonella bongori* and *Salmonella enterica* based on biochemical and serological assays further confirmed by MLST typing. The species *enterica* is further classified into six sub-species; the sup-species-I *enterica* consists of numerous serovars which causes of infections to humans and other animals. Depending upon the disease syndrome and severity of infections these serovars could further be grouped into Typhoidal causing serovars and Non-Typhoidal *Salmonella*. (Achtman, *et al.*, 2012; under free license agreement).

S. enterica strains can further be classified into serogroups and serotypes (serovars) based on the presence of surface antigens. On the basis of the O surface antigen alone (lipopolysaccharide, LPS), *S. enterica* can be classified into nearly 65 serogroups and into 2500 serovars when these strains are differentiated by presence of both O (LPS) and H (flagellar) antigens. Among these, about 1500 serovars belong to the subspecies I of *S. enterica* (Grimont and Weill, 2007; CDC, 2011-National *Salmonella* surveillance overview). Furthermore, serovars belonging to *S. enterica* subsp. *enterica* (I) can further be broadly divided into two groups as shown in Figure 1, namely Typhoidal *Salmonella*, which causes systemic typhoidal diseases and Non-Typhoidal *Salmonella* (NTS). The NTS serovars are largely associated with self-limiting gastroenteritis and bacteremia in humans and animals (Achtman *et al.*, 2012).

Clinical Relevance and treatment

Salmonella infections in humans are responsible for causing two clinical syndromes mainly typhoid or enteric fever and colitis or diarrheal disease depending upon the serovar that is responsible for infection. Serovars such as *Salmonella* Typhi, *Salmonella* Paratyphi A and B cause systemic illness in humans, with clinical manifestations including enteric fever, abdominal pain, headache, transient constipation. Prolonged infections can result in severe hepatic, spleen, respiratory or neurological damage. Untreated, these infections result in high mortality rates of 20-25% (Miller *et al.*, 2001; Parry *et al.*, 2002).

NTS serotypes such as *S. Enteritidis* and *S. Typhimurium* are the most frequent causative agents of diarrheal diseases in humans and animals across the globe. According to WHO statistical data, *S. Enteritidis* globally accounted for 65% and *S. Typhimurium* for 12% infections of all human isolates. In contrast, *S. Typhimurium* was the most frequent reported serovar among non-human isolates, accounting for 17% of the total isolates (Galanis *et al.*, 2006).

The severity of infections caused by NTS serotypes in an immunocompetent individual is more drastic and often accounts for high mortality rates. NTS infections if untreated have a fatality rate of 0.1% to 0.5 % in developed countries in patients suffering from previous gastric surgery, diabetes and HIV (Gordon 2008 and Hohmann 2001). On the other hand, the mortality rate is relatively high in third world and developing countries, accounting for nearly 24% deaths per year. Such high death rates are mostly attributable to economic factors contributing to poor hygiene conditions (Feasey *et al.*, 2012). In third world countries, children often suffer from malnutrition and are often prone to *Salmonella* infections resulting in hospital admissions at a very early age with symptoms of bloody diarrhea and bacteremia in > 20% of cases, and high rates of complications (Mandomando *et al.*, 2009 and Chimalizeni *et al.*, 2010; Feasey *et al.*, 2012).

NTS infections often result in a carrier state due to persistent colonization of the pathogen in the gut in both humans and livestock. These carrier hosts play a vital role in propagation of the diseases and act as reservoirs with high zoonotic potential as the infections are often asymptomatic, with little or no obvious clinical characteristics (Crawford *et al.*, 2010; Chausse *et al.*, 2011). As reported by previous studies, only 5×10^4 bacteria when ingested either through contaminated water or food are capable enough to cause Salmonellosis and other gastrointestinal illnesses (Wray and Sjoka 1978). However, 5% of the infected individuals develop bacteremia. Furthermore; young children suffering from pre-existing medical conditions such as malaria and malnutrition are also more frequently infected (Lightfoot *et al.*, 1990).

Treatment

Depending upon the severity of illness and physiological condition of the patient, different strategies could be adopted against the pathogen. The majority of gut-limited NTS infections often result in substantial loss of body fluid, thus in such cases it is important to maintain the electrolyte balance. Therefore, such patients are often provided with either oral or intravenous

rehydration so as to counteract the fluid loss (Hohmann 2001). In contrast, in patients with high risk factors and severe illness due to high invasion rates are often put on antimicrobial therapy (Coburn *et al.*, 2007). Outbreaks of Salmonellosis due to fecal shedding are controlled by the use of antibiotics (Lightfoot *et al.*, 1990). Antibiotics such as ampicillin, fluoroquinolones, cephalosporins (cefixime or ceftriaxone) or trimethoprim-sulfamethoxazole (TMP-SMZ) are highly efficient for treating large scale *Salmonella* infections. However, the overuse of these antibiotics has also resulted in antibiotic resistance among *S. Typhimurium*. Furthermore, multiple antimicrobial resistance has been reported in *S. Typhimurium* where >55% of total isolates were found to be multi-drug resistance (MDR) (Erdem *et al.*, 2005; Weill *et al.*, 2006,). In addition, the acquisition of phage type DT104 and other hybrid plasmids have resulted in ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline) type resistance, conferring resistance against nearly all available antibiotics and which has posed a problem in the medical as well as veterinary communities (Cloeckaert and Schwarz 2001; Antunes *et al.*, 2004; Herrero *et al.*, 2008; Herrero *et al.*, 2009).

***Salmonella* serovars and their host specificity**

Salmonella serovar host specificity refers to the differences among *Salmonella* serovars in terms of the type of infections (or lack of infection) they cause for a given host. *Salmonella* serovars exhibit specificity towards their preferred host. The preference for a particular host highlights the ability of a serovar to adapt to a particular host. Depending upon numerous factors including the surrounding environment and immunological state of the host (Thinnimitr *et al.*, 2012), the dose of infection and the biological age during which the host is infected (Kingsley and Baumber, 2000), the severity of infection caused by the same serovar varies drastically. Even after the availability of complete genome sequences of several *Salmonella* serovars, it is still unclear what mechanism make one serovar more virulent in towards a particular host while making it completely non pathogenic or less virulent into another, thus making the serovar specific towards that host.

The serovars of *S. enterica* subspecies I have been characterized into three groups on the basis of their ability to colonize various hosts and the severity of infections caused within these hosts. These include the unrestricted, host-adapted and host-restricted groups as summarized in Table 1. The serovars of the unrestricted group infect a wide range of hosts, generally causing mild, enteric diseases. Epidemiologically, these serovars represent a high risk zoonotic reservoir as they have developed mechanisms to infect different hosts and persist without necessarily evoking severe clinical symptoms (Clarke *et al.*, 1993).

Group	Serovars	Host	Infections
Unrestricted Serovars	<i>S. Typhimurium</i> <i>S. Enteritidis</i>	Humans, Poultry Cattle, Swine Mouse	Enterocolitis in humans and Swine Asymptomatic carriers in Poultry and Cattle Septicemia in mouse.
Host Adapted	<i>S. Dublin</i> , <i>S. Choleraesuis</i>	Cattle, Pigs , rarely in Humans, Mouse and Chickens	Septicemia, Enterocolitis in Cattle Fatal systemic infection in swine Bacteremia in Humans and Mouse.
Host restricted	<i>S. Typhi</i> <i>S. Gallinarum</i> <i>S. Abortusequi</i>	Humans Poultry Horses	Typhus, Diarrhea Septicemia, fatal Leads to abortions in mares

Table1. Host specific characterization of *Salmonella* Serovars (Adopted from Vikash Singh, 2013; under free license agreement).

Serovars such as *Salmonella* Typhimurium and *Salmonella* Enteritidis belong to this group. Serovars of the host-adapted group infect and cause high rates of systemic infection in their preferred hosts, but are usually excreted (shed) without significant clinical symptoms in other hosts. For example, serovars *Salmonella* Choleraesuis and *Salmonella* Dublin often cause fatal systemic infections in swine and cattle, respectively (McCuddin *et al.*, 2008). However, these are usually isolated as gastrointestinal pathogens and excreted in rodents and humans. The third group is comprised of serovars such as *Salmonella* Typhi, *Salmonella* Paratyphi,

and *Salmonella* Gallinarium/Pullorum which are restricted to infections of one specific host. These serovars have been suggested to modulate their host environment in such a manner which favors their own growth and characterised by a high tropism towards lymphatic organs of their host (Uzzau *et al.*, 2001). The serovars representing the host-restricted group are responsible for causing fatal systemic infections

Factors determining host specificity

Successful adaptation to a host by any pathogen is a complex process as it involves numerous factors and genetic determinants which are a result of large number of gene products. The very basic strategies or characteristics that have been observed whereby pathogens adapt in order to establish themselves within their host involves either acquisition of novel virulence determinants and plasmids or a tendency toward genome reduction (Brendan, 2000). Serovars such as a *Salmonella* Typhimurium, Enteritidis, Pullorum, Gallinarium Dublin and Paratyphi C are classic examples which have undergone gene deletions (Clarke *et al.*, 1993). As a result, serovars like Cholerasuis and Paratyphi C have lost the ability to replicate in the intestinal lumen of their respective host, although these successfully cause systemic infections (Chiu *et al.*, 2005). Apart from gene deletions, horizontal gene transfer has led to acquisition of virulence factors, which have contributed significantly in host adaptation of *Salmonella* serovars. Attachment to host cell surface is a pre-requisite parameter to be considered for a successful invasion of *Salmonella* within its host. Mannose-sensitive pathogenicity determinants such as FimH-like adhesins play an important role in adhesion of *Salmonella* to its host cell surface (Guo *et al.*, 2009). Allele variations or point mutations resulting in structural modification of these adhesions could be an important determining factor leading to changes in host-specificity (Guo *et al.*, 2009). For example, type I FimH adhesions are found to be expressed by unrestricted serovars which infect both mammals as well as chickens, however, type-2 FimH adhesions are expressed only by host-restricted serovars such as *Salmonella* Gallinarium (Guo *et al.*, 2009).

In addition to genetic factors, other factors such as physiological state of host cell, the availability of amino acids and the ability of one serovar to compete with others or the endogenous microbiota, can also play a role in the virulence pattern of a serovar (Tierrez and Portillo, 2005). For example, in animal infection studies, *S. Typhimurium* showed a more rapid replication in swine ileal mucosa relative to the swine-adapted serovar *S. Choleraesuis*. However, as a result of its rapid replication, *S. Typhimurium* infection also resulted in elevated and sustained immune responses leading to its faster clearance, whereas the slow-growing *S. Choleraesuis* showed reduced host immune responses, but was successfully able to disseminate, eventually leading to systemic infections in swine (Paulin *et al.*, 2007).

***Salmonella* and its host**

***Salmonella* pathogenicity islands and other virulence determinants**

Salmonella has acquired a large number of virulence genes and other pathogenicity determinants via horizontal gene transfer (Hall, 2010; Jacobsen *et al.*, 2011). The majority of the genes encoding for these virulence factors are located either within highly conserved, genomic sequences known as *Salmonella* pathogenicity islands (SPIs), or are found on stably inherited virulence plasmids such as pSLT (Jones *et al.*, 1982). At least 60 chromosomally-encoded virulence genes located on different pathogenicity islands have been reported to mediate host cell invasion and intracellular survival and replication. Thus far, at least 12 SPIs have been found in genome sequences of various *Salmonella* serovars, many of them being found in all genomes within *Salmonella enterica* subsp. *enterica*, with the exceptions of SPI-6 and SPI-7 which have only been reported in *Salmonella* Typhi (Jacobsen *et al.*, 2011). In addition to the five major SPIs in *Salmonella* Typhimurium, other virulence components such as the pSLT plasmid, fimbrial/adhesin genes and flagella also contribute significantly in determining the virulence of the pathogen (Jones *et al.*, 1982; Marcus *et al.*, 2000).

***Salmonella* pathogenicity island-1 (SPI-1)**

SPI-1 encodes gene target products responsible for host cell invasion, particularly epithelial cells, by modulating the host cytoskeleton arrangement and mediating actin polymerization. SPI-1 comprises a 40kb region located at centosome 63 (Miller *et al.*, 1995; Hensel 2004), and which harbours at least 35 genes encoding for a type-III secretion system, often termed as T3SS-1 (Kimbrough and Miller, 2002), as well as effector proteins which are injected into the host cell cytoplasm. Numerous operons located within SPI-1 encode for different structural components of a functional T3SS. The *inv/spa* and the *prg/org* operons encode the core component and needle complex proteins, respectively (Collazao and Galan 1997, Klein *et al.*, 2000; Sukhan *et al.*, 2001). The *spc/sip* operon encodes effector proteins and the translocon composed of the SipB, SipC and SipD proteins which form a pore-forming structure embedded in the host cell membrane and which transport the effector proteins into the host cytosol (Kimbrough and Miller 2002). Additionally, SPI-I also encodes for several chaperones which bind to their specific target effector proteins thereby protecting them from degradation, preventing their premature interaction and also unfolding them in a manner which allows their easy translocation through the needle complex into the host (Zurawski *et al.*, 2004; Dai and Zhou, 2004; Ruiz *et al.*, 2004).

***Salmonella* pathogenicity island-2 (SPI-2)**

Salmonella pathogenicity island-2 (SPI-2) is a 40 kb sequence region, which is divided into two segments, a 15kb smaller segment and 25kb larger segment. The 15kb smaller segment is present in both *Salmonella* species *bongori* and *enterica*, and it contains the *ttRSBCA* operon which is involved in tetrathionate reduction (Hensel *et al.*, 1999). Additionally, it also has seven open reading frames (ORFs) whose function is yet to be deciphered. Conversely, the larger 25 kb region is restricted to subspecies of *Salmonella enterica* and encodes a second T3SS, which functions to secrete effector proteins involved in enabling *Salmonella* to survive and replicate within a membrane-bound compartment within infected host cells called the

Salmonella-Containing vacuole (SCV; Hensel *et al.*, 1995; Ochman *et al.*, 1996). SPI-2 harbours four types of genes whose designations are related to their functions and play an essential role in virulence: *ssr* encoding the regulator; *ssa*, genes for T3SS-2 apparatus; *ssc* encoding the chaperones and *sse* encoding the effector proteins (Marcus *et al.*, 2000; Kuhle and Hensel, 2004).

Other pathogenicity islands

Salmonella Typhimurium harbours 3 additional pathogenicity islands, SPI-3, SPI-4 and SPI-5. These 3 pathogenicity islands have not yet been studied in detail, thus little is known about their function(s). SPI-3, is a 17kb region, acquired through horizontal gene transfer and is conserved in both *Salmonella* Typhimurium and *Salmonella* Typhi. SPI-3 encodes for *mgtCB* operon whose gene products facilitate bacterial survival in macrophages (Blanc-Potrad and Groisman, 1997). SPI-4 represents a 27kb region inserted at 92 minutes on *Salmonella* Typhimurium chromosome (Wong *et al.*, 1998). Originally, 18 open reading frames (ORFs) were identified within this island but after completion of the *Salmonella* Typhimurium LT2 genome sequence, these were re-annotated to indicate only 6 ORFs (McClelland *et al.*, 2001). SPI-5 represents a 7.6 kb region which is conserved among all *Salmonella* serovars. It encodes for the SopB/SigD effector proteins which are secreted by SPI-1 T3SS and play a role in actin polymerization. SPI-5 also encodes for *pipA*, *B*, *C*, *D* and *orfX* gene products (Wood *et al.*, 1998). The gene product of genes *pipA* and *pipB* is translocated via the T3SS-2 into the host cell and PipA have been reported to play an essential role in the development of systemic infection in mice (Knodler *et al.*, 2002). PipB is reported to localize in the intracellular lipid rafts present on the SCV membrane (Knodler *et al.*, 2003). PipC act as a chaperone for the transporter protein SigE (Knodler *et al.*, 2005; Patel and Galan, 2006).

Plasmid-encoded virulence factors

Among all *Salmonella* serovars, only a few clinically important serovars such as *Salmonella* Typhimurium, *S. Enteritidis*, *S. Choleraesuis* and *S. Dublin* harbour a serovar-specific virulence plasmid. These plasmids encode virulence-associated genes which are known to play a significant role during the later stages of infection process and contribute to the intracellular growth at sites beyond the intestine, as shown in various mice infection models (Gulig, 1990; Gulig and Doyle, 1993). Depending upon the serovar, the virulence plasmids usually range from 55 to 95kb. All serovars positive for the virulence plasmid share an 8-kb conserved region encoding 5 genes, *spvRABCD*, and which are transcribed as an operon. SpvR is a positive transcriptional regulator which regulates the expression of other genes on the operon in response to low pH and nutrient limited conditions (Guiney *et al.*, 1995). SpvB and SpvC are the only encoded effector proteins in the operon. SpvC is responsible for pro-inflammatory response of the host (Mazarkiewicz *et al.*, 2008), whereas SpvB is a cytotoxic protein whose role is related to later intracellular stages of infection process (Matsui *et al.*, 2001).

In addition to the pSLT-type plasmid, other plasmids have also been reported which harbour antimicrobial resistance genes and are usually of larger size. For instance, *Salmonella* Typhimurium has been found to harbour hybrid plasmids, such as pUO-stRV2, which is approximately 140kb in size and may have originated from the pSLT plasmid with acquisition of a complex, antimicrobial locus involved in multiple antibiotic resistance of *Salmonella* Typhimurium (Guerra *et al.*, 2002; Herrero *et al.*, 2008).

Intestinal invasion by *Salmonella*

Salmonella Typhimurium infections are generally caused by the ingestion of bacteria in contaminated food and water. Upon ingestion, the first obstacle that the pathogen has to overcome within the host is the acidic pH of the stomach. In order to protect itself against this acidic shock, *Salmonella* Typhimurium activates the acid tolerance response (ATR) which

makes the intracellular pH within *Salmonella* much higher to that of extracellular environment (Foster and Hall, 1991). The bacterium then reaches the small intestine where it transverse through the intestinal mucosal layer before adhering to the intestinal epithelial cells. *Salmonella* thrives on the Payer's patches, which are abundant with specialized epithelial M (microfold or membranous) cells, which are considered the initial or primary site for infection (Takeuchi, 1967; Jones *et al.*, 1994). *In vivo* studies have shown that *Salmonella* can be found within M cells as early as 5 min. post-infection (Schauser *et al.*, 2004; Morgan *et al.*, 2004). On adherence to M cells, *Salmonella* brings about significant physical changes in the cell. The bacterium induces cytoskeleton rearrangement at the apical surface of M cells resulting in bacterial internalization (Finlay *et al.*, 1991). However, unlike other invasive gastrointestinal pathogens such as *Shigella* or *Yersinia* which are dependent upon M cells in order to breach the intestinal epithelial barrier (Jones *et al.*, 1995; Jensen *et al.*, 1998), *Salmonella* infection/invasion of the host is independent of M cells (Lee and Falkow, 1993).

Salmonella has developed mechanisms to invade, survive and proliferate both in phagocytic and non-phagocytic cells. *Salmonella* successfully infects epithelial cells, macrophages, dendritic cells, enterocytes and neutrophils (Lara-Tejero and Galan, 2009). The pathogen employs different strategies to mediate its entry into host cells. *Salmonella* could enter either by phagocytosis, through its T3SS-1 dependent or T3SS-1 independent pathways (Collazo and Galan, 1997). Bacterial invasion characterized by T3SS-1 results in secretion/injection of SPI-I encoded effector proteins directly into the epithelial cells cytoplasm. These effector proteins bring about actin polymerization and tampers with the host cytoskeleton in the process mediating the bacterial entry by macro-pinocytosis (Galan, 1998). Some of the SPI-1 secreted effector proteins that are required for bacterial internalization are SipA, SopB, SopA, SopD, SopE and SopE2 (Raffatellu *et al.*, 2005). The effector proteins SopB, SopE and SopE2 activate Rac1, Cdc42 and Rho-family GTPases. The activation of Cdc42 and Rac1 leads to recruitment of WASP and Scar/WAVE family of proteins and these together with the

Arp2/3 complex initiates the actin polymerization (Hardt *et al.*, 1998). SipC, when secreted, anchors itself within the inner membrane of the host cell and serves as a site for nucleation and bundling of free, cytosolic actin. SipA mediates actin filament polymerization and increases the stability of the resulting actin filaments (Srikanth *et al.*, 2001; Haraga *et al.*, 2008). The role of the key effector proteins is highlighted in Table 2, which play an important role in the biogenesis of the SCV (Ramos-Morales, 2012).

In addition to SPI-1-mediated host cell invasion, fimbriae and flagellae also contribute towards *Salmonella*'s entry into epithelial cells (Chessa *et al.*, 2009). Mutations in the *tdcA* gene affect flagellar biosynthesis in *Salmonella* Typhimurium and these mutants are reported to be less invasive and show reduced growth in epithelial cells (Lim *et al.*, 2010). Fimbriae facilitate the attachment between the bacteria and the host epithelial cell by binding to the extracellular matrix of the epithelial cells (Kukkonen *et al.*, 1993; Baumler *et al.*, 1997). Another important factor which plays a role in invasion of *Salmonella* into epithelial cell is O antigen length in the lipopolysaccharide (Holzer *et al.*, 2009).

After breaching the intestinal epithelial wall, the bacterium reaches the mesenteric lymph node (MLNs) via intestinal lymph through professional phagocytic cells such as macrophage and dendritic cells (Vazquez-Torres A, *et al.*, 1999; Neiss and Reinecker, 2006). Within the MLNs, the bacterium successfully infects macrophages and triggering a response similar to that in epithelial cells to ensure its survival and replication (Alpuche-Aranda, 1994; Rydstrom and Wick, 2007). These infected macrophages then help in systemic dissemination of *Salmonella* via bloodstream to other tissues such as spleen and liver (Ohl and Miller, 2001; Worley *et al.*, 2006).

The *Salmonella*-Containing Vacuole (SCV) and intracellular survival

Following internalization, *Salmonella* establishes itself within a membrane-bound vesicle termed as *Salmonella*-containing vacuole (SCV). This unique membrane-bound compartment serves as a replicative niche for the bacterium within the cell (Garcia *et al.*, 2008). The

biogenesis of the SCV is characterized by recruitment of several members of the Rab family of small GTPases (Smith *et al.*, 2007), movement of the SCV from the plasma membrane to the perinuclear space (Ramsden *et al.*, 2007), and interactions with host cell endocytic and exocytic pathways and involvement of *Salmonella*-encoded T3SS1 and T3SS2 effectors, which play a critical role in modulation and development of the SCV.

Biogenesis of the SCV

The biogenesis of the SCV has been divided into three stages: early (10min-1h post infection), intermediate (1h-4h), and late (>4h) {figure 2 and table 2} (García-del Portillo *et al.*, 2008; Malik-Kale *et al.*, 2011). Immediately after formation, the SCV undergoes rapid membrane modifications driven by the secreted T3SS1 effector's SopB and SptP. SopB acts through its phosphoinositide phosphatase activity to activate Akt via PI(3,4)P₂ and PI(3,4,5)P₃ (Steele-Mortimer *et al.*, 2000). SopB which recruits itself on cytosolic phase of SCV, reduces the levels of PI(4,5)P₂ and phosphatidylserine resulting in reduced levels of negatively charged lipids on the SCV (Mallo *et al.*, 2008). This leads to exclusion of several Rab's and/or prevents the fusion of SCV with lysosomes (Bakowski *et al.*, 2008). SopB also recruits GTPase Rab5 to the SCV membrane, leading to recruitment of the sorting nexins SNX1 and SNX3, important regulators of membrane trafficking. SNX1 is a member of a retromer sorting complex that mediates the retrieval of receptors from the endolysosomal pathway to the *trans*-golgi network (Bujny *et al.*, 2008; Braun *et al.*, 2010). Within few minutes of *Salmonella* infection, SNX1 shifts from its endosomal localization to the site of bacterial entry. SNX1 then results in formation of spacious tubules, reducing the vacuolar size and removing the cation-independent mannose-6 phosphate (CI-M6PR) receptor from the nascent SCV (Bujny *et al.*, 2008). CI-M6PR is important for delivering soluble lysosomal enzymes to lysosomes and as it is normally excluded from SCV, therefore enabling the bacteria within SCV to survive and replicate (Garcia and Finlay, 1995). SNX3 on the other hand recruits Rab7 and

LAMP-1 to the SCV membrane and contributes towards the SCV movement to peri-nuclear region (Braun *et al.*, 2010).

The intermediate stage in the SCV biogenesis is mainly characterized by involvement of dynein, as *Salmonella* uses dynein-mediated transport along the microtubules in order to reach a peri-nuclear position adjacent to the microtubule organizing center (MTOC) (Ramsden *et al.*, 2007). This process is mediated by effector proteins both from T3SS1 (SipA and SopB) and T3SS2 (SifA, SseF and SseG) (Wasylnka *et al.*, 2008). In addition, the actin-based motor non-muscle myosin II, is also implicated in contributing to SCV positioning involving the phosphate activity of SopB (Wasylnka *et al.*, 2008). During the late phase of SCV maturation, a set of T3SS effector proteins act in a co-operative manner in order to maintain the SCV at the peri-nuclear space, and also to initiate bacterial replication within the SCV (Abrahams *et al.*, 2006; Deiwick *et al.*, 2006). At the same time, from the cytosolic surface of the SCV membrane, tubules begin to extend rapidly along microtubules towards the cell periphery, accompanied by the acquisition of various late endosomal markers such as Lamps (LAMP-1 and LAMP-2), vATPases and Rab9 and Rab7 (Steele-Mortimer, 2008).

A T3SS2 effector protein, SifA, is required for formation of the prominent tubular structures known as *Salmonella*-induced filaments (Sifs; Stein *et al.*, 1996). SifA, along with another T3SS2 effector, PipB2, leads to recruitment of two host cell proteins, kinesin-1 and kinesin binding protein protein SKIP (SifA and kinesin-interacting protein) on the SCV which play an important role in Sif formation and maturation of the SCV (Boucrot *et al.*, 2005; Dumont *et al.*, 2010).

SseJ, a T3SS2 effector protein which shares homology to glycerophospholipid:cholesterol acyltransferases (GCATs; Lossi *et al.*, 2008), is involved in SCV/Sif biogenesis and stability, as it leads to cholesterol esterification and lipid formation from the SCV, which are enriched in cholesterol (Nawabi *et al.*, 2008). The function of these *Salmonella* induced tubules still remains unclear; however, one possibility is that *Salmonella* might use these for nutritional

requirements within the SCV by intercepting host cell trafficking pathways (Mota *et al.*, 2009). However, it remains unclear whether the tubule formation takes place *in vivo*.

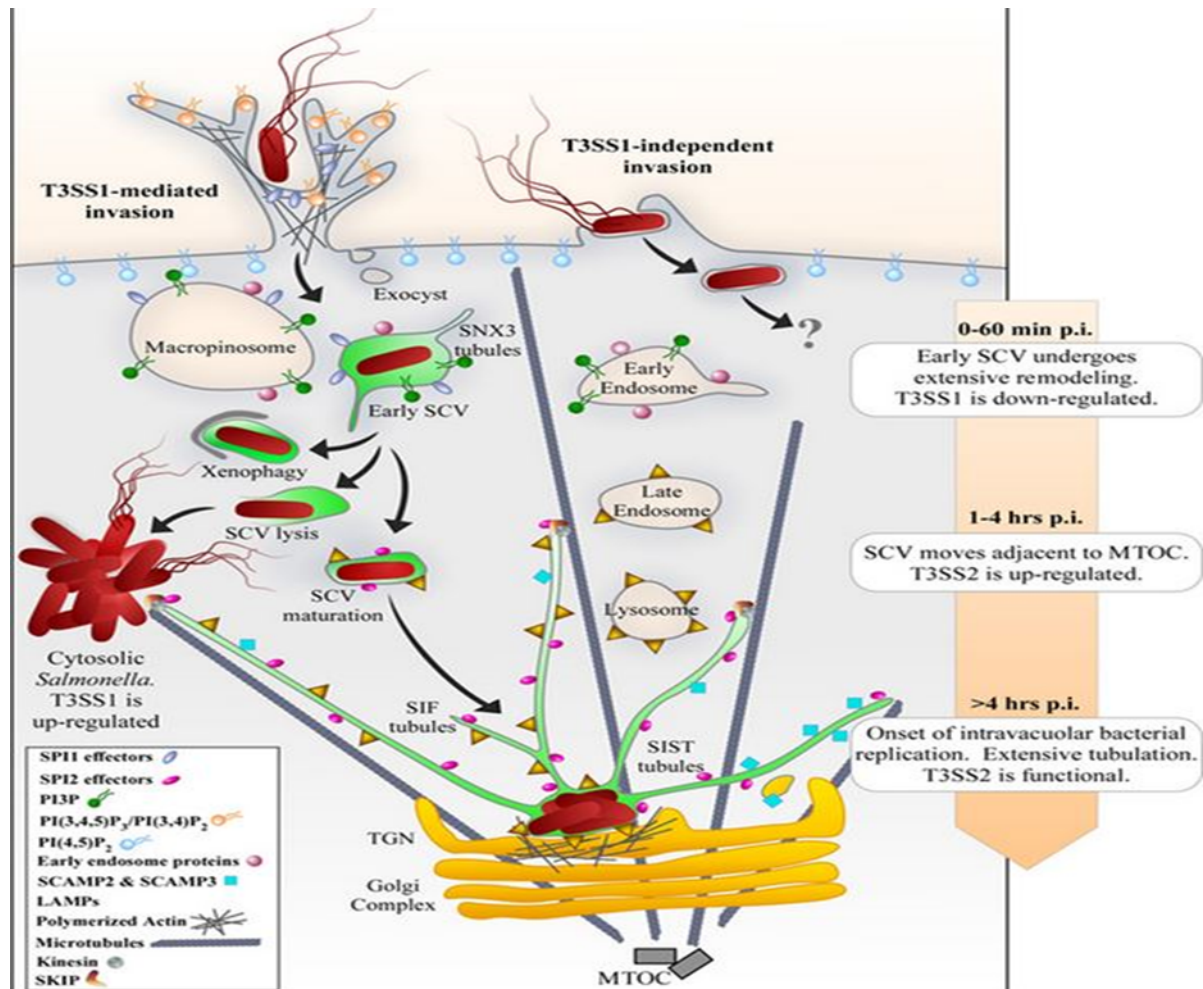


Figure 2. Biogenesis of the SCV. Upon attachment to the host cell surface *Salmonella* secretes numerous virulence effector proteins into the host cell cytoplasm using its T3SS needle complex. These effectors bring about modulation within host cell cytoskeleton thereby stimulating actin-mediated bacterial internalization by causing ruffling in the host cell membrane. The internalized bacterium develops a membrane vacuole around itself and resides within this compartment called the *Salmonella*-containing vacuole (SCV). The SCV interacts with the host endocytic pathway and matures acquiring various host cell markers such as EEA, LAMP-1, and LAMP-2 etc. The bacterium replicates within the SCV and the SCV eventually develops into a tubular network called *Salmonella*-induced filaments (sifs). (Taken from Kale *et al.*, 2011; under free license agreement).

SCV stage	Characteristics	Hostcell markers	T3SS effectors implicated
Early (<30min)	Simple vacuole or spacious phagosome.	EEA1, rab5a, rab5b, rab5c, transferrin receptor	T3SS1: SipA, SopA, SopB, SpiC/SsaB T3SS2: SpiC
Intermediate (30 min – 4 h)	Vacuole primarily in juxtanuclear position	Lamps, vATPase, rab7, rab11a, rab11b	T3SS1: SopB, SipA T3SS2: SSeF, SseG, SpiC/SsaB, SteC, SseJ/SifC
Late (>4 h)	Initiation of intracellular replication and formation of tubules (Sifs) radiating throughout cells. Microtubule and actin accumulation around juxtanuclear SCV.	Lamps, vATPase, rab7, rab9	T3SS2 PipB2, SifA, SopD2, SpiC/SsaB, SseF, SseG, SseJ/SifC, SteC,

Table 2. Stages of SCV biogenesis: Table summarizing stages of SCV biogenesis with their characteristics, host markers associated at each stage and T3SS effectors involved during the stages of SCV development and maturation.

Escape from SCV

The SCV is considered the primary survival and replicative niche for intracellular *Salmonella*; however, this may vary depending upon the host cell type where the bacteria can also be found in the cytosol. Although SCV is a replicative niche in cultured epithelial cells and macrophages, but it is not the case in fibroblasts and dendritic cells (Garcia-del Portillo *et al.*, 2008). Recent studies have indicated that in epithelial cells *Salmonella* can exhibit a bimodal lifestyle, with replication within the SCV as well as in the cytosol (Malik-Kale *et al.*, 2012). Interestingly, in the HeLa epithelial cell line, *Salmonella* shows robust growth in the host cell cytoplasm (Brumell *et al.*, 2002; Knodler *et al.*, 2010). This may not, however, be the case in macrophages where the cytosolic environment has been reported to be lethal for *Salmonella* (Malik-Kale *et al.*, 2011).

In addition, it has been reported that SCV membrane damage caused by the bacterial secretion systems, in particular T3SS2, could also lead to targeting of the bacterium for degradation *via* lysosomes through the host cell protein and organelle turnover system known as autophagy (xenophagy) (Birmingham *et al.*, 2006). It has been reported that post-invasion, a fraction of bacteria (~ 20%), show damaged SCV membranes and are exposed to the cytosol, where the bacteria are associated with ubiquitinated proteins (Birmingham *et al.*, 2006) and these ubiquitinated bacteria are then targeted to lysosomes which results in killing of bacteria (Perrin *et al.*, 2004). *S. Typhimurium* may also induce autophagy in macrophages, possibly due to effects on mitochondrial membrane morphology by the T3SS1 effector SipB which localizes to mitochondria membrane (Hernandez *et al.*, 2003).

Autophagy

Autophagy is the cellular process whereby lysosomes contribute to the degradation of intracellular compartments such as organelles, proteins and even pathogens (Mizushima *et al.*, 2008; Mizushima and Komatsu, 2011). Autophagy is an important house-keeping process for all cells as it enables the cell to maintain and balance its sources of energy under normal as well as in response towards nutrient stress conditions thus in a way maintaining the overall its cellular homeostasis (Glick *et al.*, 2010). Autophagy is also involved in cellular quality control by removal of damaged intracellular components (Hara *et al.*, 2006). Defects in the ability to perform this cellular process have been linked to protein conformation disorders, neurodegenerative disorders and myopathies (Wong and Cuervo, 2010). Additional functions that have been recently attributed to autophagy includes cell survival and response to stress (Wang and Levine, 2010), tissue differentiation (Mizushima and Levine, 2010), senescence and even anti-ageing (Wong and Cuervo, 2010), thus preventing from diseases such as cancer diabetes, liver diseases and auto-immune diseases (Glick *et al.*, 2010; Yang *et al.*, 2011; Gonzalez *et al.*, 2011). Thus, autophagy is a central, important house-keeping cellular process

which functions not only to eliminate intracellular aggregates and damaged organelles but also against intracellular pathogens.

The different steps involved in this process include, cargo-recognition, sequestration from the cytosol, delivery to lysosomes, degradation by lysosomal enzymes and recycling of the essential components of the macromolecule degraded (Yang and Klinosky, 2010a). Autophagy has been distinguished into three different types based on how the cytosolic cargo is recognized and targeted to lysosomes for degradation. The three best characterized processes which degrade unwanted/ no longer needed proteins and maintain cellular quality control are macroautophagy, microautophagy and chaperone-mediated autophagy (CMA).

Macroautophagy

This type of autophagy, involves degradation of cargo proteins which are recognized in bulk in the cytosol. Here, the cargo protein to be degraded is enclosed within a double membrane vesicle called the autophagosome (Mizushima *et al.*, 2001). Molecules such as p62 or NBR1 bind to cargo proteins through ubiquitinated residues and to Atg8/LC3, which are an important component of the autophagosome membrane (Lamark *et al.*, 2009). These autophagosomes then fuse with lysosomes which are rich in hydrolases and other enzymes required for complete degradation of the cargo. Basal levels of macroautophagy are present in all cell types and contribute towards the maintenance of cellular homeostasis (Hara *et al.*, 2006; Ruben *et al.*, 2008).

Microautophagy

In microautophagy, the cargo protein or substrate directly fuses to the lysosomes and are degraded. This process is marked by development of invaginations of the lysosomal membrane containing the cargo, which then pinches off as vesicles into the lysosomal lumen (Ahlberg and Glaumann, 1985). Microautophagy activity can be detected in many cell types; however, it is not known whether this pathway can be further upregulated under specific conditions.

Chaperone-mediated autophagy (CMA)

Chaperone-mediated autophagy is a selective type process which is responsible for degradation of cytosolic proteins bearing a penta-peptide KFERQ amino acid motif, which targets them to the lysosome for degradation (Dice, 1990). Substrate proteins that contain the KFERQ targeting motif are recognized by a cytosolic chaperone, the heat-shock cognate protein of 70kDa, Hsc70/Hsc73, in a complex with other co-chaperones (Chiang *et al.*, 1989; Kaushik and Cuervo, 2012; Cuervo and Wong, 2014). After binding, the chaperone mediates delivery of the substrate protein to the surface of the lysosome, where it binds to a receptor at the lysosomal membrane, the lysosome-associated membrane protein type 2A (LAMP-2A; Cuervo and Dice, 1996). Interaction of the substrate with LAMP-2A promotes the multimerization of this lysosomal membrane protein into a higher order complex required for translocation of substrates across the lysosomal membrane (Bandyopadhyay *et al.*, 2008). A luminal form of Hsc70/Hsc73 residing in the lysosomal lumen (ly-Hsc73) assists in the translocation of the substrate in an ATP-dependent manner (Chiang *et al.*, 1989). Upon translocation into the lumen, the substrate proteins are rapidly degraded (in 5-10 min) by a wide range of lysosomal proteases and other hydrolytic enzymes. Organelles and other large, protein complexes do not function as CMA substrates, as they have to be transported through the lysosomal membrane and LAMP-2A complex.

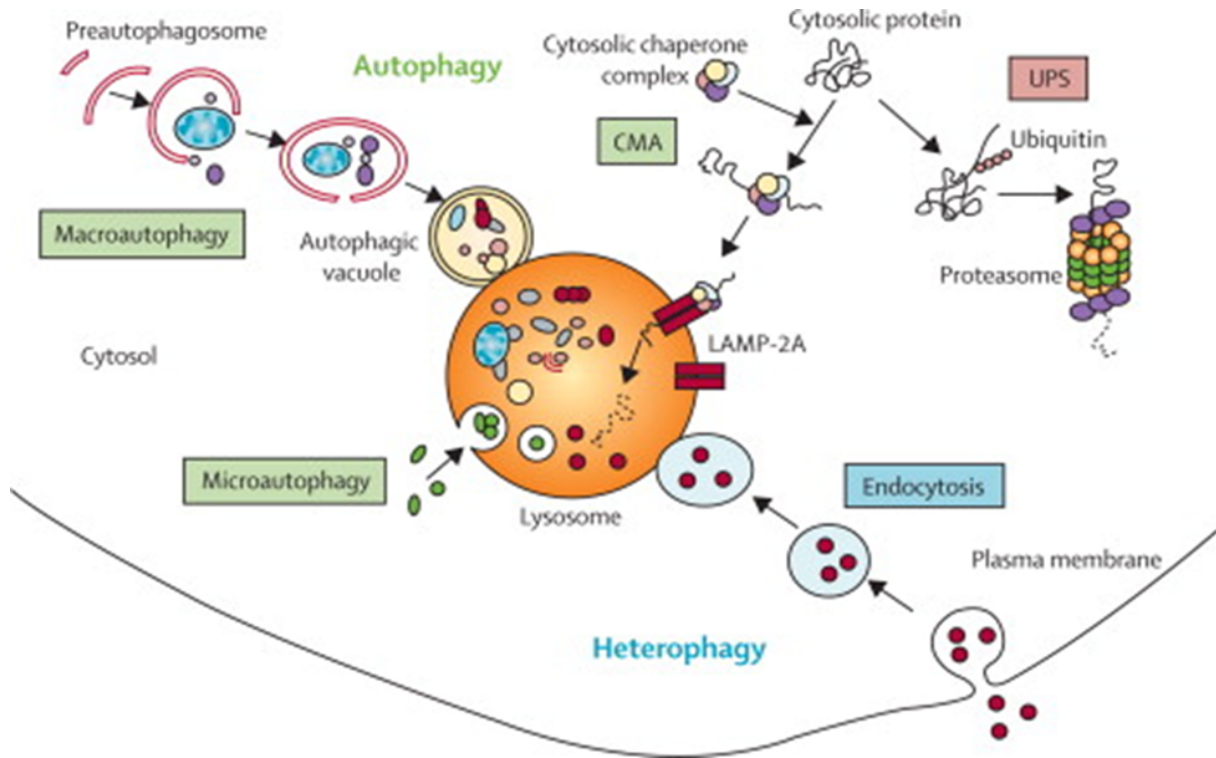


Figure 3. Proteolytic systems in mammalian cells: Substrate proteins are delivered to lysosomes from the extracellular media (heterophagy) or from inside the cell (autophagy). The best described heterophagic pathway is endocytosis. In mammalian cells three types of autophagy pathway have been described namely macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). In macroautophagy the target proteins are sequestered within a membrane bound vesicle which then fuses with lysosomes. In microautophagy, substrate proteins are internalized directly into the lysosomal membrane. In CMA, the selective substrate proteins are translocated into the lysosomes membrane by a chaperone Hsc73 where the target proteins enter into the lysosomes through a translocon formed by lysosomal receptor (LAMP-2A). **(Taken from Vicente and Cuervo, 2007; Copyright permission licence 3405891134143).**

Substrates for CMA

A basal level of CMA activity is detectable in almost all cell types, and approximately 30% of soluble cytosolic proteins contain the CMA targeting motif (Dice, 1990). Substrates targeted through this selective type of autophagy includes a heterogeneous pool of intracellular proteins including glycolytic enzymes such as GAPDH (glyceraldehyde-3-phosphate dehydrogenase), aldolase, phosphoglyceromutase, transcription factors and inhibitors of transcription factors (*e.g.* c-fos, inhibitor of NF κ B (I κ B)), vesicular trafficking proteins such as alpha-synuclein, calcium binding proteins (Annexin I, II, IV and VI), cytosolic forms of secretory proteins (alpha-2-microglobulin), as well as catalytic and subunits of proteasome, the major cytosolic protease (Massey *et al.*, 2004).

Components of CMA

Molecular chaperone complex: The heat shock cognate protein Hsc70, (also known as Hsc73) is a molecular chaperone which belongs to the heat shock protein 70 (Hsp70) family (Chiang *et al.*, 1989; Panjwani *et al.*, 1999). Due to the different nomenclature designations and isoforms, Hsc70 is hereafter referred to as Hsc73 throughout the thesis. Hsc73 is constitutively expressed and is responsible for protein translocation across membrane (Craig *et al.*, 1994). The cytosolic form of Hsc73 recognizes proteins containing KFERQ motifs and thus selectively targets the substrate proteins to lysosomes for degradation (Craig *et al.*, 1994). A number of other co-chaperones interact with Hsc73 and regulate its activity. The heat shock protein Hsp40 stimulates the ATPase activity of Hsc73 and leads to enhanced binding and also release of substrate proteins. Hsc73 interacting protein (Hip) stimulates the assembly between Hsc73, Hsp40 and substrate proteins (Hohfeld *et al.*, 1995; Suh *et al.*, 1999). Another important chaperone that plays an important is heat shock protein of 90 kDa, Hsp90. Hsp90 prevents the unfolded proteins from aggregating or refolding of substrate proteins (Richter and Buchner, 2006). The Hsp90-Hsc73 organizer protein (Hop) links Hsc73 and Hsp90 (Demand *et al.*, 1998). Hsc73 along the chaperone complex carries the target protein to the lysosomal membrane where the substrate protein binds to the lysosomal associated membrane protein 2A (LAMP-2A).

LAMP-2A: The lysosome associated membrane protein-2A (LAMP-2A), belongs to the family of membraneglycoproteins similar to LAMP-1 (Sawada *et al.*, 1993). LAMP-2A is one of the three spliced isoforms originated as result of alternative splicing of the *lamp2* gene. The three isoforms LAMP-2A, LAMP-2B and LAMP-2C, differ from one another in the aminoacid sequence representing the C-terminus region (Cuervo and Dice, 2000). Lamp-2A is an integrated membrane protein which is present as single monomers in the lysosomal membrane with a single transmembrane region. The majority of the protein lies in

glycosylated into the lysosomal lumen and a short cytosolic tail present in the cytoplasm (Eskelinen *et al.*, 2005).

Of the three isoforms, only LAMP-2A is known to have a function in the CMA process, as it acts like a receptor for the incoming substrate protein destined to be degraded via the CMA pathway (Cuervo and Dice, 1996). The chaperone Hsc73 carries the substrate protein bearing the pentapeptide sequence KFERQ to the lysosomal membrane, where the substrate protein binds to the cytosolic region of LAMP-2A (Bandyopadhyay *et al.*, 2008). The binding of the substrate protein to LAMP-2A is the rate limiting step in the CMA pathway (Cuervo and Dice, 2000c), as this results in multimerization of LAMP-2A monomers to form a translocon through which the substrate protein passes into the lysosomal lumen and is eventually degraded (Bandyopadhyay *et al.*, 2008).

The lysosomal form of Hsc73 (lys-Hsc73): A lysosomal form of Hsc73 is also present into the lysosomal lumen (Agarraberes *et al.*, 1997). Furthermore this lysosomal form of Hsc73 is stable at acidic conditions. Similar to the luminal chaperones present in mitochondria and ER which are required to pull proteins into these organelles, the Lys-Hsc73 is also believed for transport of the substrate proteins into the lysosomes which are then further degraded by lysosomal enzymes (Brodsky *et al.*, 1998; Artigues *et al.*, 2002), as CMA could be completely blocked by allowing fibroblast cells to endocytose an antibody specific for Hsc73 (Agarraberes *et al.*, 1997).

Regulation of CMA

CMA is activated maximally under stress conditions, such as oxidative stress, nutrient deprivation such as serum (and/or amino acid) deprivation and exposure to toxins (Wing *et al.*, 1991; Cuervo *et al.*, 1999; Kiffin *et al.*, 2004). The selective nature of CMA is considered beneficial during prolonged starvation, as it favours the degradation of non-essential proteins thus providing amino acids for cell survival. Furthermore, under oxidative stress conditions it facilitates the selective removal of damaged proteins.

LAMP-2A levels in the lysosomal membrane play an important role in regulating the functional activity of CMA (Cuervo and Dice, 2000b). Levels of LAMP-2A at the lysosomal membrane are tightly regulated as this membrane protein undergoes rapid cleavage by membrane metalloprotease and cathepsin A (Cuervo *et al.*, 2003). LAMP-2A degradation decreases under conditions facilitating CMA activation (nutrient stress or oxidative stress) thereby resulting in a doubling of the half-life of LAMP-2A. However under low CMA activity, LAMP-2A is found in cholesterol rich domain within the lysosomal membrane. Upon treating mammalian cells with cholesterol extracting drugs such as methyl- β -cyclodextrin results in increment of LAMP-2A levels in the lysosomal membrane, thus activating CMA (Kaushik *et al.*, 2006).

Pathological relevance of CMA

Chaperone mediate autophagy is a selective form of autophagy where intracellular proteins containing a recognition motif are specifically selected and degraded in lysosomes (Dice, 1990). Thus any malfunction or inability of a cell to perform CMA has been linked to the onset of several human disorders (Arias and Cuervo, 2011).

Impairments in CMA activity have been associated with several neurodegenerative diseases such as Alzheimer's, Parkinson and Huntington's diseases (Cuervo *et al.*, 2004; Wang *et al.*, 2010; Arias and Cuervo, 2011). With increasing age of an individual the CMA activity declines, due to reduction of LAMP-2A levels at the lysosomal membrane (Cuervo and Dice, 2000a) This age dependent dysfunction in CMA activity contributes to lysosomal storage disorders, different types of toxic neuropathies and diabetes (Cuervo *et al.*, 1999; Sooparb *et al.*, 2004; Venugopal *et al.*, 2009; Welsch *et al.*, 2010).

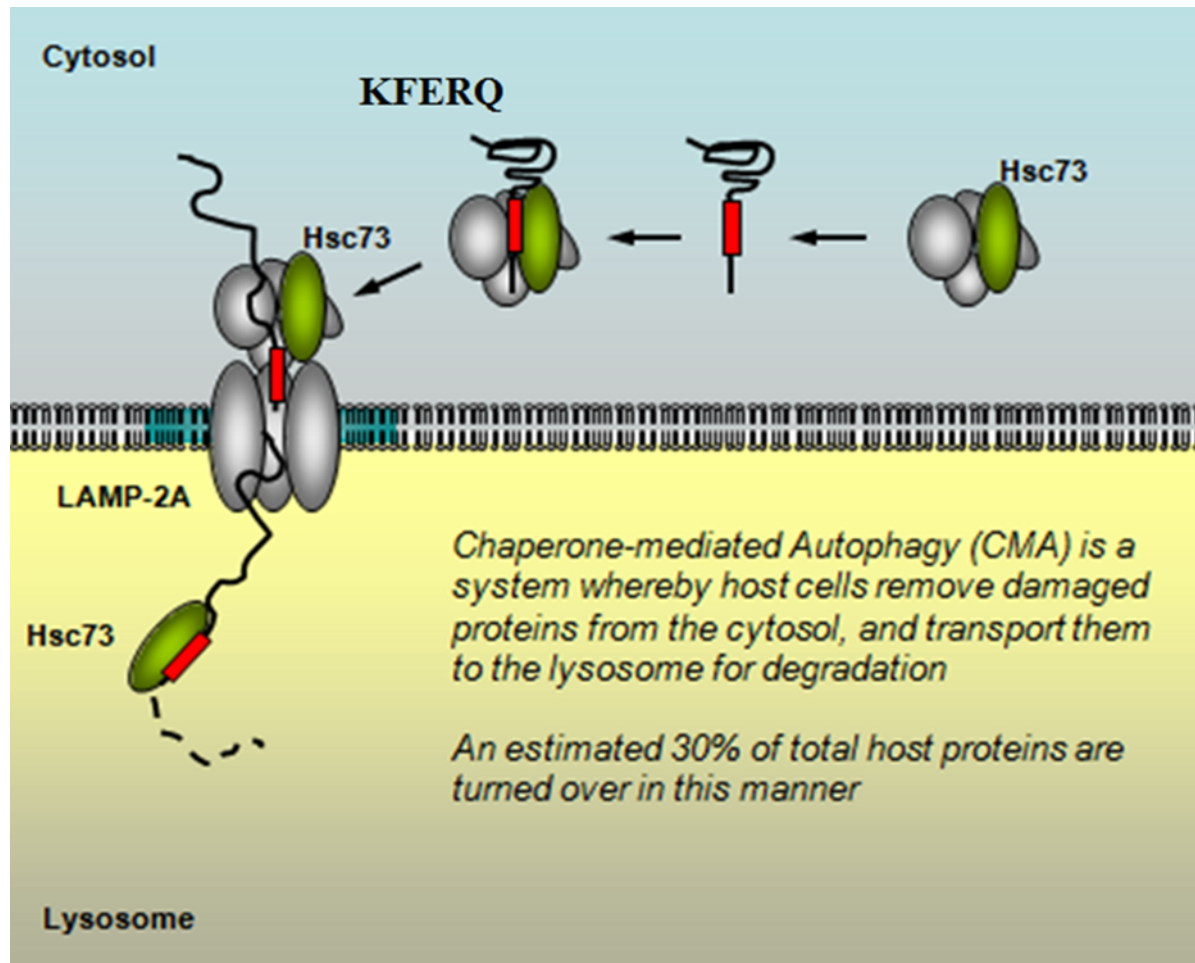


Figure 4. Schematic representation of CMA pathway. Substrate proteins bearing the KFERQ amino acid motif are recognized by heat shock cognate protein 73 kDa (Hsc73) in the cytosol, which then transfers the substrate protein in association with other co-chaperones to the cytosolic face of LAMP-2A on the lysosomal membrane. LAMP-2A multimerizes with binding of substrate protein and it acts as a trans-locon through which the substrate protein is delivered into the lysosomal lumen with the help of lysosomal Hsc73. Within the lysosome the substrate protein is degraded by hydrolytic enzymes and lysosomal proteases into amino acids to be re-used by the cell. An estimated 30% of total cell proteins are turned over *via* the CMA pathway (Cuervo and Wong (2014)). (Figure modified with permission from Karsten Tedin, 2011 unpublished)

Furthermore, upregulation in basal levels of CMA activity has been described for survival and proliferation of cancer cells due to upregulation of LAMP-2A levels in the lysosomal membrane (Saha, 2012; Cuervo and Wong, 2014). Thus both reduction and overexpression of CMA activity has been linked to various diseases signifying the importance of regulating the CMA activity at basal levels in all cell types.

AIMS

Salmonella spp. are facultative, intracellular pathogens, which infect and proliferate within intestinal epithelial cells, macrophage and other cell types. Post-infection or -invasion of host cells, *Salmonella* remains within a membrane-bound intracellular compartment referred to as the *Salmonella*-containing vacuole (SCV) (Holden, 2002; Garcia-del Portillo *et al.*, 2008; Steele-Mortimer, 2008; Bakowski *et al.*, 2008; Eswarappa *et al.*, 2010; Lahiri *et al.*, 2010). Although establishment of the SCV has been well characterized, little is known about how *Salmonella* obtains nutrients and substrates required for growth within this compartment (Lundberg *et al.*, 1999; Yimga *et al.*, 2006; Bowden *et al.*, 2010; Dandekar *et al.*, 2012; Bowden *et al.*, 2014).

Interestingly, over 90% of clinical isolates of *Salmonella* Typhi, the causative agent of human Typhoid, have been shown to be auxotrophic for amino acids (Virgilio and Cordano, 1981). Furthermore, Hoiseth and Stocker (1981) showed that *aroA* strains of *Salmonella* Typhimurium are highly attenuated in mice models of infection. *aroA* strains are unable to synthesize chorismate, the precursor for aromatic amino biosynthesis (Neidhardt *et al.* 1996; Eisenreich *et al.*, 2010) However, these authors also showed that wild-type levels of virulence can be rescued by providing mice with metabolites not involved in aromatic amino acid biosynthesis but which also require chorismate. These studies suggest that *in vivo*, *Salmonella* must have access to host-derived sources of amino acids for intracellular growth. We were therefore interested to know how *Salmonella* could acquire nutrients within the SCV in this situation.

As *Salmonella* should be dependent upon host cell proteins and pathways for accessing sources of nutrients within the host, I therefore chose to examine more closely some of the host proteins present at the SCV membrane which might be involved in providing peptides, which could provide a source of both carbon sources as well as amino acids.

Furthermore, for characterization of various host proteins and compartments which might interact with the SCV, it was necessary to purify *Salmonella*-containing vacuoles from infected host cells. In an effort to avoid chemical or physical interactions which could affect and possibly disrupt the labile SCV membrane, I therefore developed a relatively simple, rapid method to purify intact SCV's using paramagnetic nanoparticles.

Methods

Molecular biology methods

Plasmid preparations from *E. coli* and *Salmonella*

A single bacterial colony was inoculated into 5 ml L-broth (Lennox, 1955) containing the appropriate antibiotic and incubated at 37°C with aeration until the cultures reached an optical density at 600 nm (OD₆₀₀) of approximately 2. Plasmid DNA from bacterial cultures was isolated using the Qiagen Plasmid Midi Kit (Qiagen, Inc.) according to the manufacturer's instructions.

Precipitation of DNA

Approximately one-tenth volume of 3M sodium acetate (pH 5.5) and 2.5 volumes of ice cold 100% ethanol were added to the sample containing the DNA. The samples were mixed and were allowed to precipitate at -20°C overnight. The following day, the samples were centrifuged at 18500 rpm for 60 min. at 4°C. The resulting pellets were washed twice with 70% ethanol, air-dried and re-suspended in sterile, deionised water.

Determination of concentration of nucleic acids

The concentrations of DNA were determined either by Spectrophotometer or Nanodrop Spectrophotometer (PeqLab) according to the manufacturer's instructions. For quantification of the amount of DNA present, the following formula was used:

$$1 \text{ unit of absorbance of dsDNA at } 260 \text{ nm } (A_{260}) = 50 \text{ } \mu\text{g/ml dsDNA}$$

The purity of the DNA samples was determined by calculating the ratios of the absorbance at A₂₆₀ to A₂₈₀. DNA samples are considered adequately pure if $1.8 \leq \text{absorbance at } A_{260} / \text{absorbance at } A_{280} \leq 2.0$. A value less than 1.8 indicates contamination with proteins or with aromatic substances like phenol, while a value greater than 2.0 indicates possible contamination with RNA.

Electrophoresis and detection of nucleic acids on agarose gels

Agarose gel electrophoresis was used for the routine analysis of DNA. Depending upon the size of DNA fragment to be separated a 0.8- 1.2% agarose gels were casted using 1X Tris-borate buffer (TBE) containing 0.5 µg/ml Midori Green Advance. DNA samples mixed thoroughly in DNA loading buffer were allowed to run in agarose gels at 100 V (generally, 10V/cm).

Polymerase chain reaction (PCR)

Desired DNA fragments were amplified using the thermostable DNA polymerases. The DNA fragments up to 2kb were amplified using the standard BioTherm Taq polymerase (Rapidozym). The PCRs were set up and performed according to the following specifications.

Table 3. PCR reaction mixture pippeting Scheme

Reagent	Amount
Forward primer (10 pmol/ µl)	1 µl
Reverse primer (10 pmol/ µl)	1 µl
dNTP mix (2mM)	5 µl
10X Reaction buffer	5 µl
MgCl ₂ (50mM)	1.5 µl
DNA template	10-100 ng
HPLC water	Up to 50
DNA polymerase	1 unit

Table 4. PCR thermal cycling programme

Phase		Temperature and time
Denaturation		94°C, 3 min
Denaturation		94°C, 1 min
Annealing	35 cycles	45 – 60°C, 1min
Elongation		72°C, 1-2 min
Extension		72°C, 10 min

Large DNA fragments of size more than 2kb were amplified using Abgene Extensor Hi-Fidelity PCR master mix (Thermo Scientific) according to the supplier's instructions.

Microbiological methods

Preparation of bacterial stocks

Bacterial laboratory stocks were streaked to Lennox agar with or without antibiotics and incubated overnight at 32°C or 37°C, depending on the strain. 5 ml of Lennox broth was inoculated with a single colony and grown until an OD₆₀₀ of 2-3. 1 ml of this bacterial culture was transferred to a cryo-tube containing 300 µl of a sterile 80% glycerol solution, vigorously mixed and preserved at -80°C.

Preparation of competent bacteria for transformation with plasmid (rapid method)

A single bacterial colony was inoculated in 5 ml of LB containing appropriate antibiotic and grown on a shaker (200rpm) at either 30 or 37°C depending on the strain. Upon reaching an OD₆₀₀ of 2-3, the bacterial culture was transferred to 1.5 ml Eppendorf tubes. The bacteria were pelleted by centrifugation at 13500rpm, 5 min and supernatant discarded. From this step onwards, the entire procedures were done on ice. The pellets from two tubes were resuspended in 1 ml of 10% ice cold glycerol and centrifugation was repeated with decreasing speeds of 6000x g, 3000x g, 2000x g and 1500x g for 5 min each at 4°C. The resuspended cells from two tubes were pooled prior to last round of centrifugation. The pellet was finally

resuspended in 400 µl of 10% glycerol and aliquots of 100 µl in pre-frozen 1.5 ml Eppendorf tubes were stored at -80°C.

Electroporation

Prior to electroporation, the electrocompetent cells were thawed on ice. 1-2 µl of the prepared DNA was added on to the thawed electrocompetent cells and the mix was transferred to a 2 mm electroporation cuvette. Using the EasyjecT Prima (peqLab), the mix was pulsed at 2.5 KV according to the standard protocol. The samples were immediately resuspended in 1 ml of LB without any antibiotics and incubated for 1 hour at room temperature. After this recovery period, transformants were selected by plating the diluted samples on selective medium.

Generation of bacterial mutants

All *S. enterica* serovar Typhimurium strains used in this study were derivative of the wild-type strain SL1344 (Hoiseth and Stocker, 1981). Deletion mutants were first constructed by directed, non-polar gene deletion/replacement with a kanamycin-resistance cassette in strain *S. enterica* serovar Typhimurium LT2 according to the method of Datsenko and Wanner (2000). After verification of the chromosomal location of the kanamycin-resistance cassette/gene deletion by PCR amplification of the insertion site/antibiotic cassette, bacteriophage P22 lysates were prepared. The gene deletions were then transduced into the wild-type SL1344 strain by bacteriophage P22 transduction with selection for kanamycin resistance. Phage P22-free clones were selected as single colonies taken from Green plates onto which independent, kanamycin-resistant clones had been streaked from cultures grown in L-broth with selection for kanamycin resistance. A second PCR verification for the correct gene deletion/chromosomal flanking regions of the P22 transductants was performed prior to preparation of bacterial stocks.

Multiple deletions were constructed sequentially after elimination of the kanamycin-resistance cassette by introduction of the FLP-recombinase expressing plasmid, pCP20 (Cherepanov and Wackernagel, 1995). After screening for loss of the kanamycin-resistance,

plasmid pCP20 was eliminated by growth at 37°C. Subsequent deletions were introduced by bacteriophage P22 transduction as above. The list of mutant strains, are summarised in Table 7. The sequences of all the primers used in this study are listed in Table 9.

Preparation of bacteriophage P22 stocks

After PCR verification of the deletion of chromosomal genes and replacement with the kanamycin-resistance cassette, it was necessary to transfer the gene deletion/replacement region into the virulent, isogenic strain used for further studies. The transfer of the genetic regions were performed by bacteriophage P22 generalised transduction using a derivative of P22, HT105/1, which harbours a mutation in the integrase gene and is unable to form lysogens, but remains capable of packaging both viral and chromosomal DNA (Schmieger and Buch, 1975). Donor bacteria were inoculated in 6 ml L-broth containing 10 mM MgSO₄ and 5 mM CaCl₂ and grown to OD₆₀₀ approx. 2 at 37°C. 5 to 10 µl of bacterial culture was transferred to a pre-warmed sterile test tube containing 6 ml of 0.7% soft agar, 10 mM MgSO₄ and 5 mM CaCl₂ and maintained at 70°C before adding bacterial culture. The mixture containing soft agar and bacteria was poured onto a LB agar plates and ket at room temperature for 10 minutes in order to allow hardening of the soft agar. P22 particles (100 µl) were spotted onto the soft agar and allowed to soak into the plates containing bacteria. Once the phage mixture has dried; the plates were incubated overnight at 37°C. The following day, cleared zones on the soft agar plates were collected using a sterile spatula and transferred to a 15 ml polypropylene tube (Falcon). 1 ml L-broth containing 10 mM MgSO₄ and 5 mM CaCl₂ and 1 µl of chloroform were added in to polypropylene tube and incubated overnight at 4°C to allow diffusion of the bacteriophage out of the agar. The following day, the tubes were centrifuged at 7,500 rpm 15 minutes at 4°C to remove the agar and cell debris. The clear supernatants were transferred to eppendorf tubes and stored at 4°C for further usage. 10 µl of

chloroform/ml supernatant was added into each phage stock to prevent bacterial growth and maintain sterile stocks of bacteriophage.

Transduction of *Salmonella* with phage P22

The recipient bacterial strain was cultured to OD₆₀₀ approx. 2 in 6 ml L-broth containing 10 mM MgSO₄ and 5 mM CaCl₂ at 37°C. 2 to 5 µl of the donor P22 phage lysate was further added to 100 µl of the bacterial culture and incubated at 37°C for 20 minutes. EGTA was added with a final concentration of 20 mM to chelate the metal ions necessary for phage infection to stop the reaction. The volume of the mixture was brought to 1 ml with L-broth containing 20 mM EGTA and 100 µl aliquots of the mixture was plated onto L-broth agar plate containing the appropriate antibiotic. Transductants were further screened on Green plates for the absence of lysogens and infecting phage (Sternberg and Maurer, 1991). Infected bacterial colonies due to lysis of bacterial cells are indicated by a pH change as seen by a change in the media to dark blue/green, whereas uninfected colonies maintain a pale green color.

Growth curves and growth on plates

Bacteria were routinely grown in L-broth or M9 defined glucose minimal medium containing 50 µg ml⁻¹ histidine. Where indicated, M9 minimal medium was supplemented with phenylalanine at 50 µg ml⁻¹, casamino acids at 1% (w/v), or heat-inactivated foetal calf serum at 10% (v/v). Dialysed casamino acids were prepared by dialysis of a 20% (w/v) solution of casamino acids in dialysis tubing (Serva Membra-Cell MWCO 3500, Heidelberg) against sterile, distilled water. Where used for selection of strains or plasmids, carbenicillin was present in the media at 100 µg ml⁻¹, chloramphenicol at 15 µg ml⁻¹, kanamycin at 50 µg ml⁻¹, and tetracycline at 20 µg ml⁻¹. For generation of growth curves, strains were inoculated at 10⁵ cfu in 0.1 ml medium into replicate wells of a 96-well plate and growth was monitored by

determination of the optical density at 600 nm at 15 min. intervals over 24 h during incubation with shaking at 37°C on a heated Synergy HT microtiter plate reader (BioTek).

Cell culture methods

Maintenance and cultivation of eukaryotic cell lines

Epithelial cell lines were maintained in DMEM/Ham's F-12 salts (1:1) cell culture medium with heat inactivated fetal bovine serum (FBS) at 10% (v/v). Human (THP-1) macrophage cell lines were maintained in Iscove's Modified Dulbecco's Medium (Iscove's/IMDM) supplemented with 10% FBS. All cell lines were incubated at 37°C with 5% CO₂ with the cell culture medium replaced each two to three days. Confluent cell culture monolayers were passaged by detachment with trypsin/EDTA treatment. On the day of passage, the cell culture medium was removed and the cells were washed once with phosphate buffered saline (PBS; Biochrom). The PBS was removed and replaced with one culture volume of trypsin/EDTA (0,5%/0,2% w/v in PBS) and incubated 5 to 10 minutes at 37°C. The detached cells were transferred to a sterile, 15 ml centrifuge tube and the cell suspensions diluted 1:1 with cell culture medium containing FBS. The cells were collected by centrifugation in a swinging bucket rotor (Sigma 3K30) at 155 x g for 5 min. at 25°C. The supernatants were removed by aspiration, and the resulting cell pellets were resuspended in fresh medium at the original cell culture medium volume. Flasks or well plates were seeded at empirically determined dilutions depending on the cell line and the desired cell concentrations for maintenance (passage) or experimental cultures. The cells lines were maintained for up to ten passages, then replaced by fresh cell cultures from liquid nitrogen stocks.

Cryopreservation of eukaryotic cell lines

Stocks of each cell line were preserved in liquid nitrogen in the appropriate cell culture medium supplemented with 10% dimethylsulfoxide (DMSO). At passage, 1 ml of the cell pellet re-suspensions in cell culture medium was added to 0.1 ml of DMSO, mixed gently,

and placed immediately to ice. The cryogenic vials with the cells were first stored one to three days at -80°C and then transferred to liquid nitrogen for long-term storage.

Gentamicin protection/invasion assays

The gentamicin protection assay or invasion assay is frequently used to study eukaryotic cell-pathogen interaction as an infection model (Shaw and Falkow, 1988; Elsinghorst, 1994; Edwards and Massey, 2011). Briefly, the respective cell line was seeded into 24-well plates with 1 ml of the appropriate cell culture medium. The cells were incubated at 37°C in 5% CO₂ until a confluent cell culture monolayer was achieved, approximately 2×10^5 cells per well in the 24-well plate format. One hour prior to infection the cell culture medium was replaced with fresh medium.

The day before the invasion assay, bacterial strains were streaked from the frozen -80°C stocks to a Lennox agar plate and incubated overnight at 37°C. The following day, single colonies were inoculated into L-broth and incubated at 37°C with shaking until the bacterial culture had reached the late log growth phase, corresponding to an optical density at 600 nm (OD₆₀₀) of approximately 2-3. One ml of this bacterial culture was centrifuged and the resulting cell pellet was suspended in 1 ml of cell culture medium. A 1:10 dilution of the resuspension was made and the OD₆₀₀ was again determined and the initial bacterial counts per ml of suspension were calculated according to the assumption that an OD₆₀₀ of 1 corresponds to approximately 1×10^9 *Salmonella* cfu/ml. From this resuspension, the bacteria were diluted in cell culture medium to a concentration of approximately 10^6 cfu/ml, and 100 µl of this stock was used to inoculate each well of a 24-well plate containing approximately 2×10^5 eukaryotic cells to yield a ratio of bacteria:eukaryotic cells (multiplicity of infection, MOI) of ≤ 1 . Appropriate dilutions of the bacterial culture used for the infections were also plated onto L-broth agar to calculate the actual number of colony forming units used to infect the eukaryotic cells.

Immediately after infection, the cell culture plates were centrifuged at 250 x g for 10 minutes at room temperature to initiate infection (synchronization of infection). The plates harboring the infected cells were incubated for 60 min. at 37°C in 5% CO₂ to allow the bacteria to penetrate the cells. 60 min. post-infection, 50 µg/ml gentamycin was added to the plates for an additional 60 min. to kill all remaining extracellular bacteria that had not penetrated the host cells. After one hour of co-incubation in the presence of 50 µg/ml gentamycin (2 h post-infection), the medium was replaced by fresh medium containing 10 µg/ml gentamicin. At 2, 4, and 24 hours post-infection, cells were washed with 1X PBS and lysed by addition of 0.5 ml of 0.1% Triton X-100 in deionized, distilled water. Various dilutions of the resulting cell lysates were plated directly onto L-broth agar plates and colony forming units (cfu) were counted after overnight incubation at 37°C. The percentage of bacteria that were able to invade the eukaryotic cells was calculated as the ratio of cfu at 2 hours post infection relative to the actual cfu used to infect the cells. The intracellular replication was determined using the ratio of cfu at 24 hours post infection and cfu at 2 hours post infection. The assays were repeated at least three times for each strain and each cell line.

Invasion assays with inhibitors

Epithelial cells (HEK293T and LoVo) were pre-treated with the various inhibitors at the indicated concentrations in cell culture media 4 h prior to infection. The inhibitor was maintained throughout the course of the assay. In these assays, cells were infected with the indicated *Salmonella* strains at a MOI of approximately 5 into 24-well cell culture plates (Corning). 30 min. post-infection, cells were washed with fresh medium containing 10% FCS, and were incubated in cell culture medium containing 50 µg/ml gentamycin (Sigma) and the specific inhibitor. After 1 h of incubation, cells were washed with pre-warmed 1X PBS and were incubated into cell culture medium containing 10 µg/ml gentamycin plus the same specific inhibitor for the remainder of the experiment. At the indicated time points post infection, the cell were dissolved/lysed using freshly prepared 0.1% triton X-100 (Sigma-

Aldrich) in water followed by plating of released content on L-broth agar plates containing appropriate antibiotics to determine the intracellular cfu. The ratio of cfu at 4 h relative to the actual input cfu was used to determine the percentage of invasive bacteria.

Table 5. List of chemical inhibitors used to block various host cellular pathways

Compound	Source	Working conc.	Description
3-methyl-adenine (3-MA)	Sigma-aldrich	10 mM	Macroautophagy inhibitor
cyclohexamide (CHX)	Sigma-aldrich	200 µg/ml	CMA inhibitor
ammonium chloride (NH₄Cl)	Sigma-aldrich	10 mM	Prevents acidification of lysosomes
Anti-Hsc73 antibody	13D3 (abcam)	10 µg/ml	Inhibits CMA by blocking the activity of Hsc73.

Cell Transfections

The short-hairpin RNA (shRNA) plasmids against LAMP-1, LAMP-2A and negative control scrambled RNA constructs were purchased from S.A. Biosciences/Qiagen. Stable transfections of HEK293T cells with shRNA plasmids (300 ng/100 µl) targeting LAMP-1 or LAMP-2A was performed using HiPerFect transfecting reagent according to the manufacturer instructions (Qiagen, Cat. No. 301702) with cells grown in 24-well cell culture plates. Stably transfected cells were selected by adding puromycin (5 µg/ml) into the cell culture media after 2 days of transfection. The reduced expression of LAMP-1 and LAMP-2A was confirmed by Western blotting of whole cell lysates of transfected cells. The confirmation by Western blotting was performed immediately after puromycin selection and was also repeated before cryopreserving the selected cells for storage. Selected cells were maintained in cell culture medium containing puromycin, and were subsequently passaged till passage number 5,

thereafter the stable cell line were either cryopreserved in liquid nitrogen or used for experimental purposes.

b) Plasmids pCDNA 3.1-KFERQ-DsRed sequence and pCMV-GAPDH-HT were stably transfected into HEK293T cells using HiPerFect transfecting reagent as above. HEK293T cells were seeded into a 24 well cell tissue culture plates onto 12cm² sterile coverslips (Roth). Mixture containing plasmid and transfecting reagent were added onto cells the same day in presence of serum free medium. The cells were incubated at 37°C, 5% CO₂ for 3 days in cell culture medium with neomycin (5µg/ml) selection. Stable transfections were confirmed by visualizing the cells under fluorescence microscopy as red fluorescent cells. The transfection efficiency was estimated by comparing fields under phase contrast and red fluorescence emission wavelengths. Selected cells were maintained in Cell culture medium containing neomycin, and were subsequently passaged till passage number 5, thereafter the stable cell line were either cryopreserved in liquid nitrogen or used for experimental purpose.

***Salmonella*-Containing Vacuole (SCV) Purification**

Overview of the method

The method used for isolation of pathogen-containing phagosomes is rapid, easy and gentle so as to isolate *Salmonella*-Containing vacuoles (SCV) with intact membranes. The first step in the process employs labelling of bacteria with paramagnetic nanoparticles. This was achieved by co-incubating bacteria with carboxyl-coated nanoparticles. The labelled bacteria were then collected onto a 0.2 µm sterile filter to remove unbound particles which passed through the filter. After a wash, the filter was inverted and bacteria were recovered by backwashing with 1X PBS. These labelled bacteria were then used to infect cells.

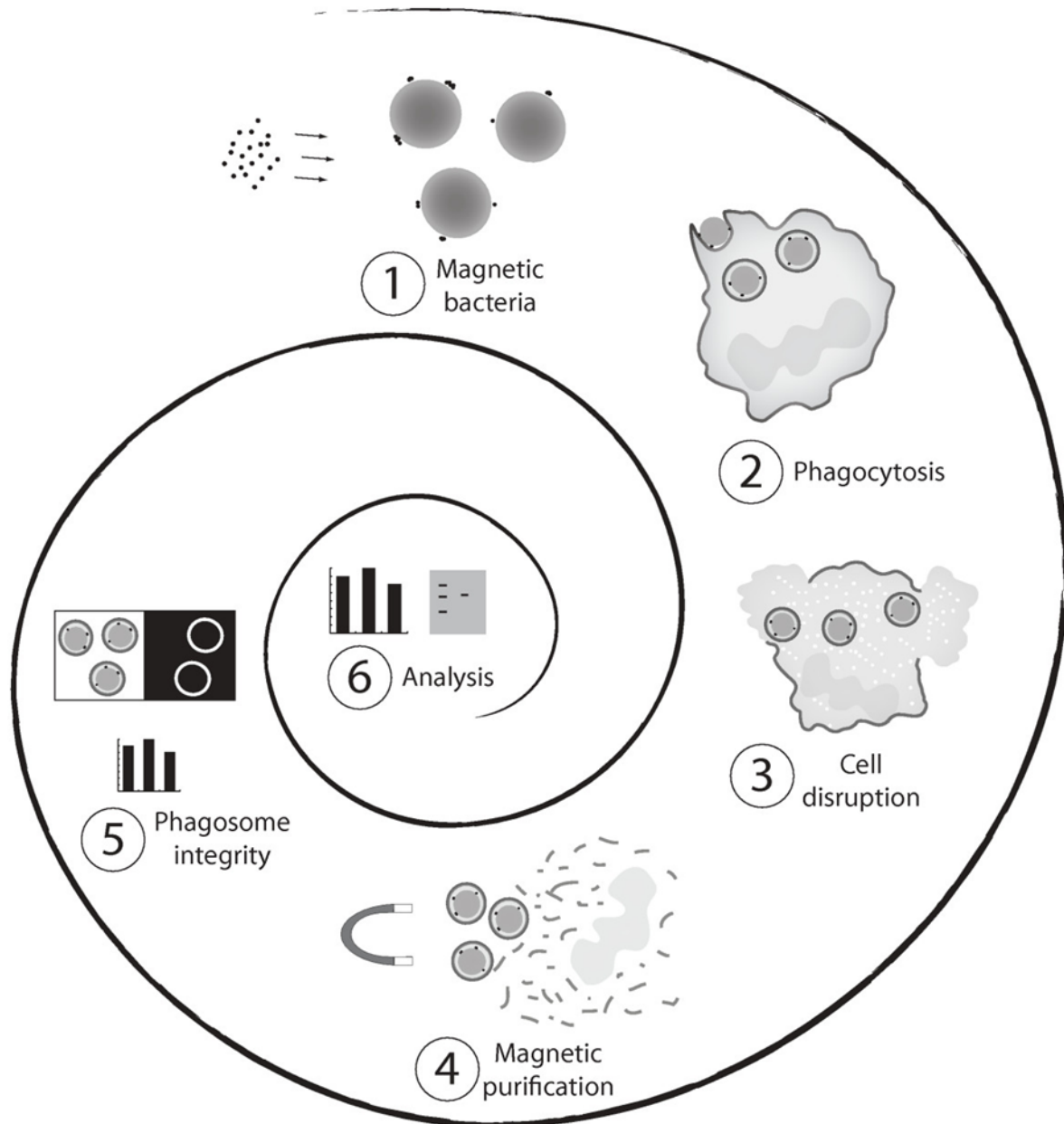


Figure 5. Schematic representation for the isolation of bacteria containing phagosomes. **1)** Bacteria are made magnetic by incubating *Salmonella* with carboxyl coated paramagnetic nanoparticles. **2)** These labelled bacteria are used to infect macrophages in a synchronized manner under centrifugation. **3)** The host cells are lysed after 24hrs p.i. using a lysis buffer (0.1% Triton X-100 in ddH₂O). **4)** In presence of an external magnetic field the pre-labelled bacteria within the phagosome is collected. The collected cell lysate is then subjected for analysis in order to check the phagosome membrane integrity by microscopy **5)** or proteomic analysis using Western blotting **6)**. (Adapted from Lonnbro *et al.*, 2008; under free liscense agreement).

After a desired time point post-infection, the THP-1 cells were lysed using a lysis buffer which caused disruption of the host cells but without affecting the intracellular *Salmonella*. Application of an external magnetic field was used to collect the pre-labelled bacteria. The collected cell lysates were washed and subjected for further analysis either by confocal microscopy or Western-blotting as described in detail in the following section.

Labeling of *Salmonella* with magnetic nanoparticles

From a stock solution of 3 mg/ml of carboxyl-coated, paramagnetic cobalt nanoparticles of diameter 10 - 50 nm (TurboBeads, Zurich), a 0.5 ml aliquot was sonicated to disperse aggregates, followed by centrifugation at 1000 x g for 30s. Following centrifugation, 0.1 ml of the resulting supernatant was diluted 1:10 in sterile, deionized water prior to use. *Salmonella* strains grown in L-broth as described above were collected from 1 ml of culture by centrifugation in a microfuge at 16,000 x g for 5 min. The resulting bacterial pellets were resuspended in PBS and incubated in the presence of a 5:1 ratio of nanoparticles: bacteria at 37°C for 20 min. with shaking. The bacterial suspension was then filtered through a 0.2 µm filter fitted to a 5 ml syringe. The filters were washed twice with two volumes of PBS, then the filter was inverted and the bacteria recovered in 1 ml of PBS collected into a sterile microfuge tube. This was then used either for infection or processed for Electron microscopy.

Infection and purification of SCV

THP-1 human macrophage cells grown in a 6-well cell culture plate were infected at a cell density of 10^6 cells/well with the nanoparticle-labelled *Salmonella* (MOI = 5) and incubated at 37°C, 5% CO₂. 24 h post-infection, the cell culture media was removed, infected cells were washed twice with PBS then lysed by addition of 1 ml/well of cell lysis buffer (0.1% Triton X-100 in water). The resulting cell lysates were then placed in a magnetic field (Single Place Magnetic Stand, Ambion, USA) and the supernatant was carefully removed and discarded, and replaced by 1 ml of a 1X PBS/2% sucrose solution. The buffer was again removed and

replaced by 0.1 ml of the same buffer to concentrate the SCVs, which were then used either for staining with antibodies or subjected to SDS PAGE and Western blotting.

Microscopy methods

Co-localization studies

Cells were grown to a confluency of about 75% in 24-well plates containing sterile, 12 mm diameter glass cover slips. These were then infected at an MOI of 5 with wild-type *S. Typhimurium* strain SL1344 or other *Salmonella* serovars harboring plasmid pGP704gfp (Vazquez-Torres *et al.* 1999). At the times indicated post-infection, cells were washed with pre-warmed 1X PBS and fixed in 4% paraformaldehyde in PBS for 15 min at 4°C. Fixed cells were washed twice with 1X PBS and permeabilised with 0.1% Triton X-100 in PBS for 5 min. at room temperature, and again washed three times with 1X PBS. The infected cells were then blocked with 1.5% BSA in PBS for 1 h at room temperature under shaking conditions. The cells were then incubated with primary antibodies in blocking solution for either 1 h or overnight at 4°C. Cells were then washed twice with 1X PBS and incubated with the appropriate fluorescently-conjugated secondary antibodies for 1 h at room temperature. Coverslips were washed three times with 1X PBS and mounted inverted using Mowiol (Sigma) on glass slides and visualized using a Leica SP-2 laser confocal scanning microscope (LCSM) using a 63X oil immersion objective. All primary and conjugated secondary antibodies used are listed in Table 11 and Table 12.

Co-localization with CMA substrate (GAPDH) and KFERQ targeting sequence

HEK293T cells stably transfected with the pCMV-GAPDH-HT plasmid were seeded in 24 well cell culture plates containing sterile coverslips at a cell density of 2×10^5 cell/well. Cells were incubated with culture medium containing 500 nM TMR-HT ligand (Promega) at 37°C, 4 hrs prior to infection with *Salmonella*. 24 h post-infection, cells were fixed with 4% paraformaldehyde in PBS. The fixed cells were mounted with Mowiol and observed using confocal microscopy (LCSM Leica SP-2).

HEK293T cells stably transfected with the plasmid pcDNA3.1-KFERQ-DsRed2 were seeded onto sterile coverslips, infected with GFP-expressing *S. Typhimurium* strains at an MOI of 5. 24 h post-infection, the infected cells were fixed using 4% PFA, washed, and incubated with 1.5% BSA in 1X PBS containing DAPI (1:500) for 1 hr at room temperature. After three washes with 1X PBS, the coverslips were mounted onto glass slides using Mowiol. These were visualized using confocal microscopy (LCSM Leica SP-2).

Microscopic analysis for purified SCV's

Isolated SCV's were incubated for 1 hr with specific primary antibody as mentioned in 1.5ml centrifuge tubes (Eppendorf) and placed in a magnetic stand. The solution opposite to the magnetic stand face was removed and the SCV's were gently washed with 1X PBS/2% Sucrose solution, followed by incubation with the appropriate secondary antibody in the same tube for 1 hr. These were then again washed and mounted onto glass slides for visualization using confocal microscopy (LCSM Leica SP-2).

Transmission electron microscopy (TEM)

THP-1 cells were infected for 20-24 h with either labelled or non-labelled *Salmonella*, and then fixed by addition of fixative solution (1.5% PFA and 1.5% glutaraldehyde in 0.15 M sodium cacodylate buffer, pH 7.4). The fixed cells were incubated at RT for 1 h and were subsequently post-fixed for 2 h at 4°C in 1% osmium tetroxide in sodium cacodylate buffer, and dehydrated in a series of ethanol steps, and further processed with acetone for Epon embedding. Sections were cut with a microtome and mounted on Formvar coated copper grids. The sections were post-fixed with uranyl-acetate and lead citrate and examined under the electron microscope.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Analytical polyacrylamide gel electrophoresis in sodium dodecyl sulphate was performed as described by Laemmli (1970). Samples were heated for 5 min at 100°C in protein loading buffer and volumes of 5-10 µl were loaded onto a 10% SDS-polyacrylamide gel cast in a

Amersham multi-gel apparatus. Electrophoresis was performed at 33 mA. Once the bromophenol blue dye front reached towards the end of the gel, the electrophoresis was stopped.

Western Blotting

Purified SCV's. Isolated SCV's were collected by centrifugation, followed by resuspension in X ml of Laemmli (6X) buffer and boiling. Equal volumes were loaded onto a 12% SDS polyacrylamide gel and electrophoresed as described above. Post-electrophoresis, the proteins were transferred to nitrocellulose membrane (GE Healthcare) using a submarine blotting method. The resulting membranes were blocked by incubation in a solution of 3% skim milk powder in 1X TBS with shaking for 1hr. The blocked membranes were washed three times using 1X TBST (Tris Buffered Saline with 0.1% Tween-20) for 5 mins each. The membrane was then incubated with the appropriate primary antibodies diluted in blocking buffer at 4°C overnight with shaking. The following day, the membranes were washed with 1X TBST and incubated with HRP-conjugated secondary antibodies for 1 hr. Signals were revealed using ECL detection system kit (ThermoScientific Pierce).

LAMP-2A and LAMP-1 knockdown cell lines. HEK293T cells, transfected with either anti-LAMP-2A or anti-LAMP-1 shRNA plasmids or scrambled RNA control plasmid were lysed with 0.1% TritonX-100 in milliQ water. The cell lysates were collected using centrifugation and were heated in Laemmli buffer for 5 mins at 90°C. These were then loaded onto SDS gels as described above and processed for Western Blotting as described for SCV preparations above. The knock-down of gene expression was confirmed by Western blotting using specific antibodies and developing the membrane using HRP-conjugated secondary antibodies using the ECL detection kit, as described above.

Statistical Analysis and Software

Statistics

1. Microscopic images in the results section are a representative images of at least 25 images per condition performed in duplicates from at least three independent experiments.
2. The graphs representing intracellular growth ,inhibitor assays and invasion rates are a result of data derived from at least three independent experiments and represent mean+- std with statistical significance addressed with Mann-Whitney U-test (ns=non-significant : $p > 0.05$, **: $p < 0.01$, ***: $p < 0.001$) as performed with Graphpad prism statistical software.
3. The western blotting images representative cropped images from the original full length developed membranes of at least three independent experiments and arranged in one panel for better characterization.
4. For quantification of microscopic images, at least 25 images per condition in duplicates from at least three independent experiments were counted to plot the graphical representation of the data.

Software

1. **GraphPad Prism and SigmaPlot:** GraphPad Prism version5 was used for preparing all the graphs and statistical analysis of the data as indicated in the figures legends. The graphs representing the growth curves of wild type *Salmonella* strain and the peptide dependent mutant strain were prepared using the SigmaPlot 11.0 Statistical software
2. **Image J** (<http://rsb.info.nih.gov/ij/index.html>) : Western blot gels were quantified using Image J software. The graphs are a representative of 3 individual experiments pooled together as arbitrary values of Intensities.

3. **Paintshop Pro and AdobePhotoshop:** Images representing growth on plates were assembled together in one panel for better characterization and representation using the Paint Shop Pro Software. All the other modifications (labelling, arrow heads etc) in the images were performed using the AdobePhotoshop 7.0.1 software.
4. **PlagScan:** (<http://www.plagscan.com>). Plagiarism scan was performed using the PlagScan online Software. The report generated is as follows:-

“116 matches from 14 sources, of which 14 are online sources. *Plagiarism level:*
4,5% Results of plagiarism analysis (excluding references section) from 2014-08-01
06:36 UTC”

A detailed documented report is deposited as both .pdf and Microsoft Word versions at the Institute for Microbiology and Epizootics, Freie Universität Berlin with Dr. Karsten Tedin.

Results

The primary pre-requisite for any intracellular pathogen in order to grow and replicate successfully with-in its host is to evolve specific mechanisms to access nutrients with-in its host. Furthermore, modulations in nutrient availability, in particular of ‘Carbon’ source with-in the host has been reported to influence and regulate the expression of various virulence genes and factors of the pathogen (Poncet *et al.*, 2009; Bouguenec and Schouler, 2011). Hence a deeper understanding about the accessibility of nutrients from the host for bacterial replication and survival is important for greater understanding of host-pathogen interactions as it would serve as a potential landmark in development of therapeutic strategies against the pathogen without affecting the host.

I. Growth analysis of *Salmonella* wild-type and peptide-dependent mutant strains

After infection of host cells, *Salmonella* Typhimurium resides within a membrane-bound compartment within the host cells known as the *Salmonella*-containing vacuole (SCV). This intracellular compartment has been suggested to be limiting for nutrients (Appelberg, 2006; Dandekar *et al.*, 2012). Despite these presumably poor growth conditions, *Salmonella* survives and replicates within this isolated compartment, indicating that the bacterium has access to intracellular or possibly extracellular sources of nutrients (Rajashkar *et al.*, 2008; Garcia-del Portillo *et al.*, 2008; Schroeder *et al.*, 2011.). Recently, a number of studies have also shown the importance of sugar utilization pathways as well as other metabolic and catabolic pathways involved in intracellular growth of *Salmonella* (Yimga *et al.*, 2006; Eisenreich *et al.*, 2010; Bowden *et al.*, 2010; Fuch *et al.*, 2012; Hofreuter *et al.*, 2012; Steeb *et al.*, 2013; Bowden *et al.*, 2014).

However, as noted in the Introduction, clinical isolates of *S. Typhi* are nearly always amino acid auxotrophs, and the loss of virulence due to the lack of chorismate in *S. Typhimurium aroA* mutants, is regained when the folate deficiency is alleviated, indicating that the bacteria obtain aromatic amino acids within the host (Hoiseth and Stocker, 1981; Hölzer and Hensel

2012). These prior studies suggest that intracellular *Salmonella* obtain at least amino acids in some form or another from the host.

Recent studies in our laboratory have shown that in cell culture models of infection, the lack of either sugars (glucose and/or myo-inositol) or amino acids, or both, do not affect the intracellular growth of a wild-type strain of *Salmonella* Typhimurium. As shown in Fig. 6. the intracellular growth of wild-type *Salmonella* Typhimurium is not effected both in human epithelial (Fig. 6 a) cells and in human macrophages (Fig. 6 b) even after starving both the cell lines for over 20 hrs with the specific nutrients. These results imply that intracellular *Salmonella* have access to an alternative source of carbon units other than the sugars normally present in cell culture media. We were therefore interested in determining the possible sources of these nutrients to the bacteria residing within the SCV.

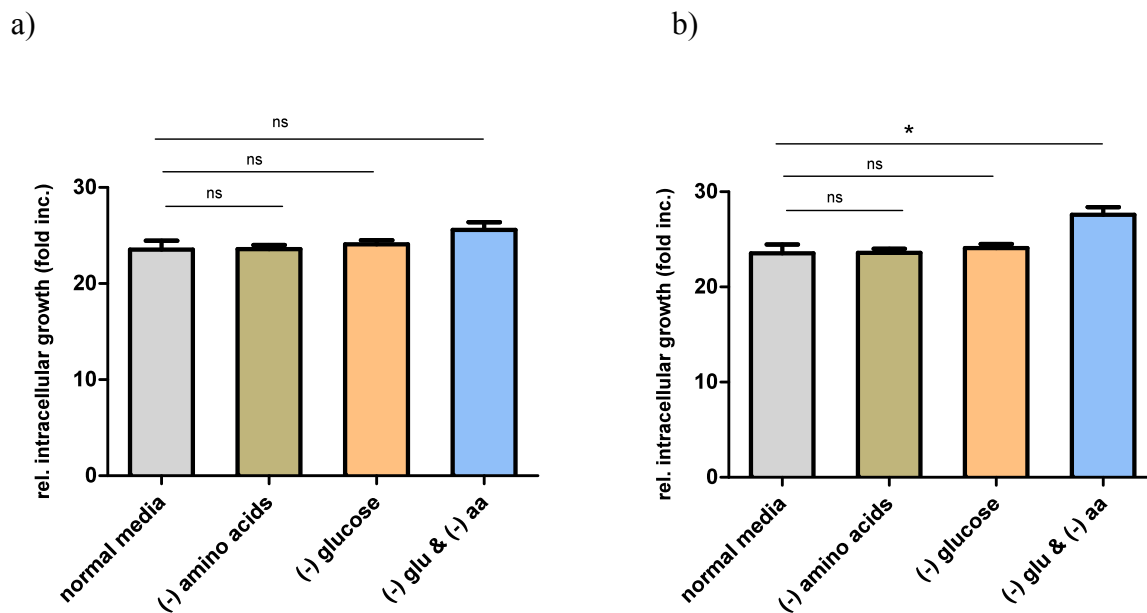


Fig. 6. Intracellular growth of wild-type *Salmonella* Typhimurium under different nutrient conditions. Shown is the intracellular fold increase of the wild-type *Salmonella* at 24 h post infection in (a) LoVo human intestinal epithelial cells and (b) human macrophages when the cells are deprived for the indicated nutrients 24 h prior to infection. The data shown are the means and standard deviations of three, independent assays, where $p > 0.05$ is considered non-significant (n.s.), *, $p < 0.05$; as determined with Mann-Whitney U-test.

Salmonella acquires amino acids through multiple pathways which include *de novo* biosynthesis, uptake of free amino acids thorough the various amino acid transporters present

in the membrane of *Salmonella*, or through the uptake and catabolism of small peptides. Notably, amino acids (and peptides) also serve as carbon sources (reviewed in McFall and Newman, 1996). Indeed, one of the most frequently used bacterial growth media for *Escherichia coli* and *Salmonella* Typhimurium is Luria-Bertani or LB medium, which contains no fermentable sugars which can be used as carbon sources for these Gram-negative bacteria (Sezonov *et al.*, 2007). As the intracellular growth of *S. Typhimurium* showed no obvious defects in the absence of either sugars or free amino acids (Fig. 6), we therefore considered the possibility that intracellular growth may, at least in part, be dependent upon access to host-derived peptides.

As noted above, prototrophic *Salmonella* are able to synthesise or take up free amino acids. In order to determine a role for host-derived peptides in intracellular growth, we constructed a mutant of *Salmonella* which is defective in both the uptake of the free aromatic amino acids (phenylalanine, tryptophan and tyrosine) and *de novo* biosynthesis of phenylalanine. Deletion of the genes encoding the transport systems involved in uptake of aromatic amino acids (Δ aroP, Δ pheP, Δ tyrP and Δ mtr) and biosynthesis of phenylalanine (Δ pheA), confers a strict dependence upon peptides as the only source of phenylalanine for growth.

As shown in Fig. 7, the mutant strain (7752) shows growth similar to that of the wild-type in growth medium containing amino acids in the form of oligopeptides such as L-broth or defined M9 minimal glucose medium containing casamino acids. However, the mutant does not show growth in either defined M9 minimal glucose medium containing phenylalanine, 1% bovine serum, or cell culture medium containing the full complement of amino acids and 10% bovine serum. These results verify that the mutant strain is not capable of biosynthesis or uptake of phenylalanine and further indicates that proteins present in bovine serum do not serve as a source of peptides to support its growth. *Salmonella* does not express extracellular proteases, thus it is unable to degrade large proteins, explaining the lack of

growth in the presence of inactivated bovine serum. Furthermore, like *E. coli*, *Salmonella* cannot transport peptides larger than 5 to 6 amino acids in length into the bacterial cytosol

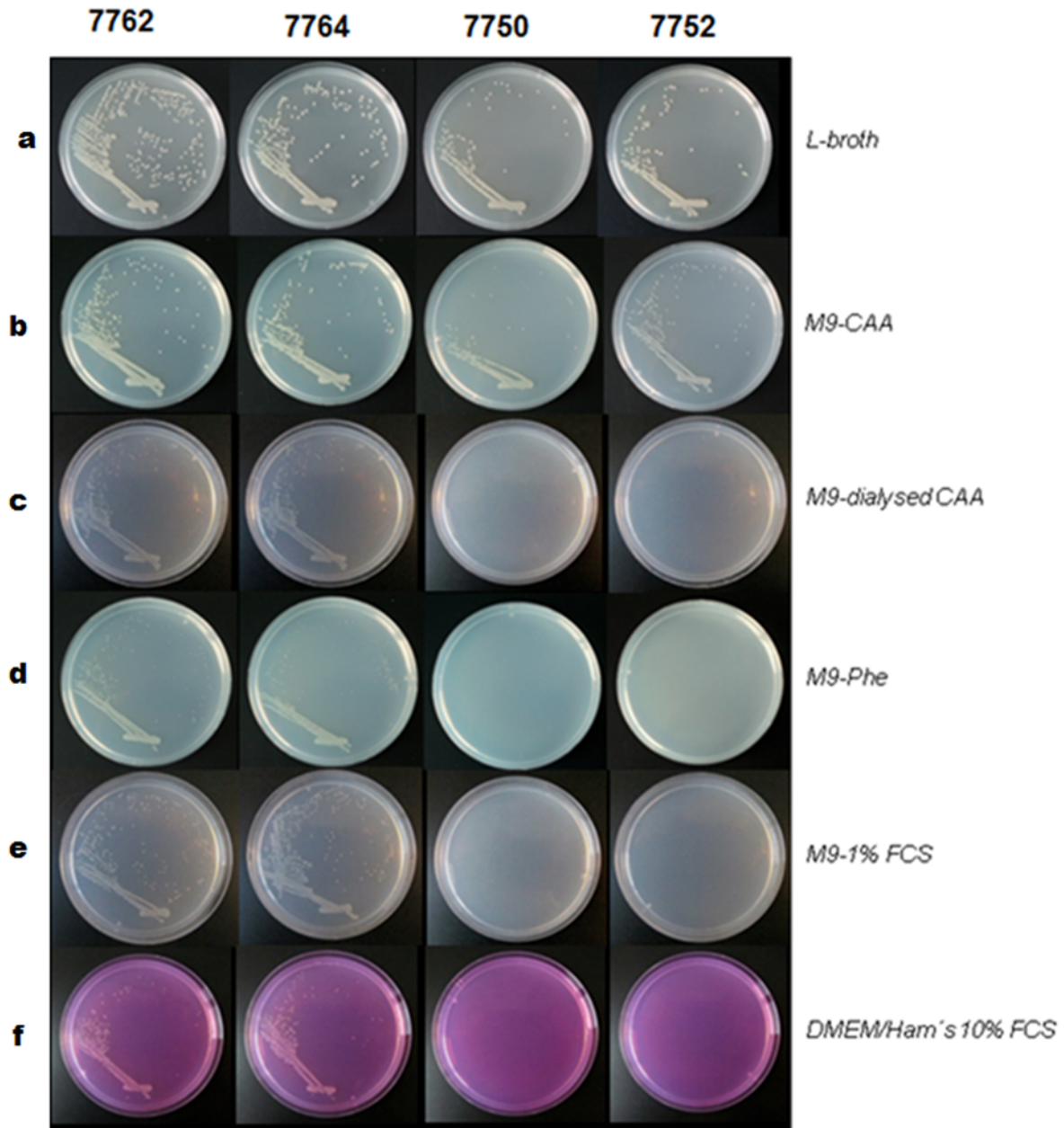


Fig. 7. Growth characteristics on solid media. Shown are the growth patterns of the strains indicated at the top of the column on the media indicated to the right: L-broth; M9 minimal medium supplemented with 0.2% casamino acids; M9 with 0.2% dialysed casamino acids; M9 with 50 μ g/ml phenylalanine; M9 with 1% fetal calf serum (FCS); and DMEM/Ham's F12 cell culture medium agar with 10% FCS. All M9 minimal basal media contain glucose and 50 μ g/ml histidine to satisfy the histidine auxotrophy of strain SL1344. Strains: 7750 - SL1344 Δ aroP Δ pheP Δ tyrP Δ mtr Δ pheA(pACYC184); 7752 - SL1344 Δ aroP Δ pheP Δ tyrP Δ mtr Δ pheA(pMHEtetCDH); 7762 - SL1344(pACYC184); 7764 - SL1344(pMHEtetCDH).

through any of the known peptide uptake systems (Payne and Smith, 1994). Consistent with this, we compared the growth of both wild-type and the peptide-dependent mutant in defined M9 minimal glucose medium containing casamino acids which had been dialysed in order to remove peptides smaller than 3.5 kDa. As seen in Fig. 8, the mutant does not grow when supplemented with dialyzed casamino acids/peptides, indicating a strict dependence on small peptides as a source for nutrition to sustain growth.

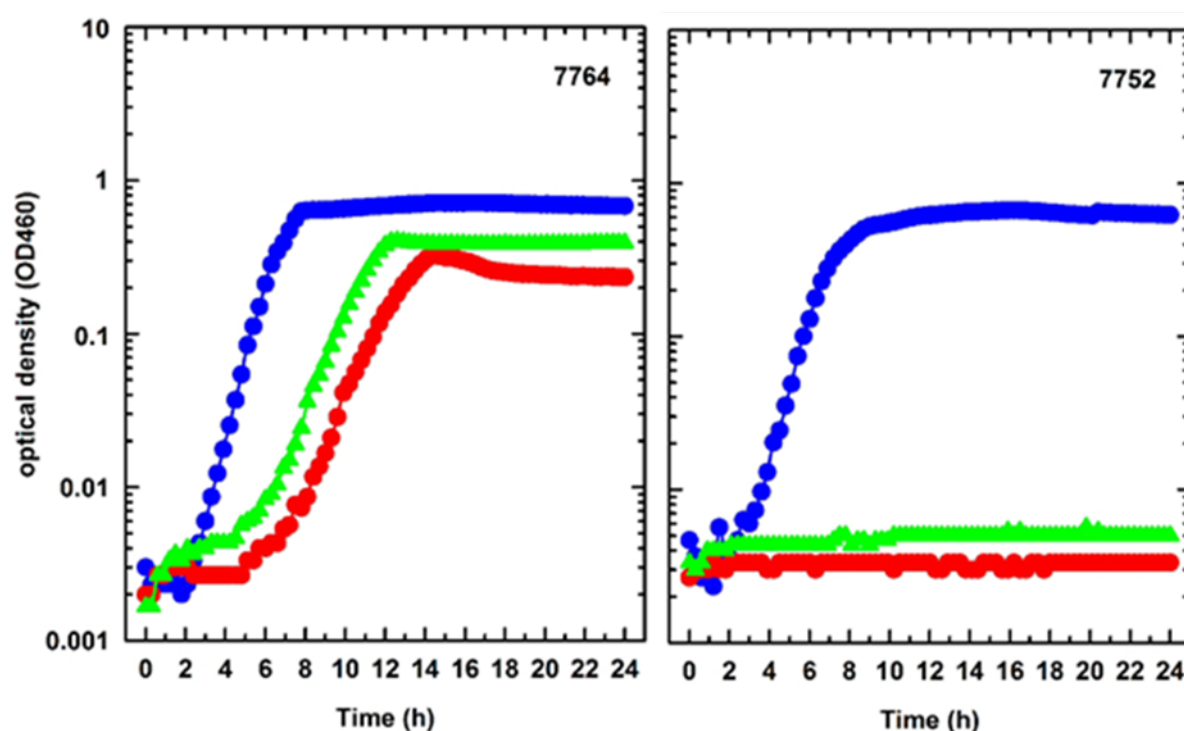


Fig. 8. Growth characteristics of the *Salmonella* $\Delta aroP \Delta pheP \Delta tyrP \Delta mtr \Delta pheA$ mutant strain. Shown are the growth characteristics of the wild-type (panel A, strain 7764) or mutant strains (panel B, strain 7752) grown in M9 glucose minimal medium broth supplemented with 1% casamino acids (blue), 1% casamino acids dialyzed to remove peptides < 3.5 kDa (green), or cell culture medium containing the full complement of amino acids and 10% serum (red). The results shown are representative of duplicate, independent trials.

In addition to determination of growth on agar-based media, we also examined growth of the wild-type and peptide-dependent mutant strains in liquid, broth cultures. As shown in Fig. 8, consistent with the plate tests, the mutant strain showed no growth in defined M9 minimal glucose media containing dialysed casamino acids nor in cell culture media with the full complement of amino acids and 10% inactivated bovine serum. Growth in the same glucose

minimal medium base containing complete (non-dialysed) casamino acids showed growth similar to the wild-type strain. As shown in Fig. 7 and 8, the wild-type strain of *Salmonella* showed growth under all conditions tested. These results verified that the *Salmonella* $\Delta aroP$ $\Delta pheP$ $\Delta tyrP$ Δmtr $\Delta pheA$ mutant strain was strictly dependent upon small peptides for growth as a source of phenylalanine, both in the presence of phenylalanine as a free amino acid, and in the presence of serum proteins in cell culture media.

Having established the mutant strains requirement for small peptides for growth in bacterial or cell culture media, we then compared the intracellular growth characteristics of the peptide-dependent mutant strain with the wild-type strain after infection of host cells. We performed invasion (infection) assays in both epithelial and macrophage cell lines and determined the intracellular growth over 24 h. As shown in Fig. 9, in contrast to the growth defects seen in bacterial or cell culture medium, the peptide-dependent mutant showed intracellular growth rates comparable to the wild-type strain at all times post-infection, suggesting that oligopeptides are available to *Salmonella* within the SCV.

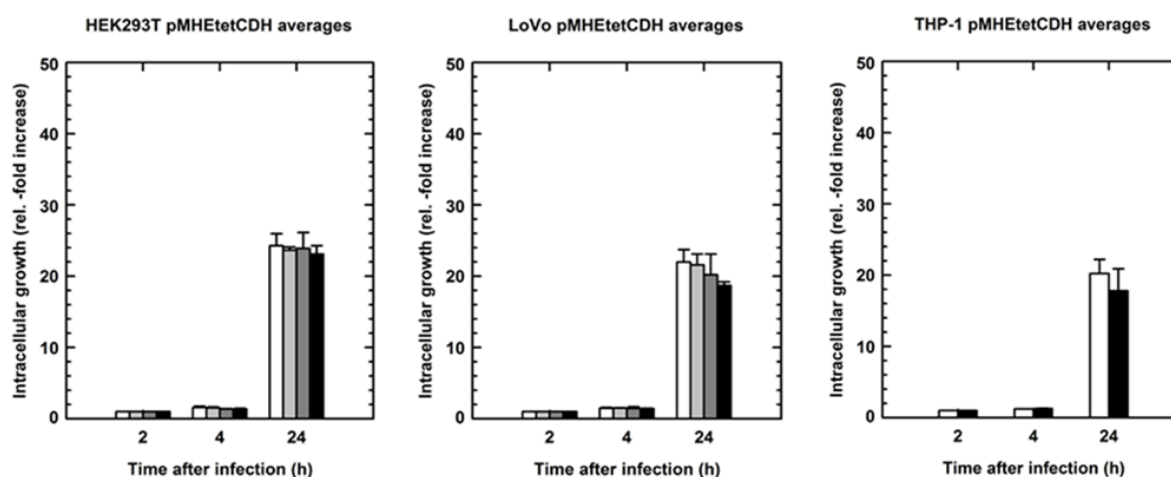


Fig. 9. Intracellular growth characteristics of the *Salmonella* $\Delta aroP$ $\Delta pheP$ $\Delta tyrP$ Δmtr $\Delta pheA$ strain in epithelial and macrophage cell lines. Shown is the relative intracellular growth over 24 h of the wild-type (left two bars) or mutant strain (right two bars) harbouring either a control plasmid (white and dark grey bars, resp.) or CDH-expressing plasmid (light grey and black bars, resp.). (A), HEK293T human kidney epithelial cell line; (B) LoVo human intestinal epithelial cell line; and (C), THP-1 human macrophage cell line. The data shown are the means and standard deviations of three, independent assays.

Alternatively, it has been reported that the SCV acidifies within a short time post-infection (Rathman *et al.*, 1996; Yu *et al.*, 2010). The intracellular growth of the mutant may therefore have been due to the spontaneous conversion of prephenate to phenylpyruvate, a reaction occurring under acidic conditions, which circumvents the lack of chorismate mutase activity due to loss of *pheA* (Davis, 1953; Katagiri and Sato, 1953). To eliminate this possibility, the strains shown in Figs. 7, 8 and 9 also harbour a plasmid encoding a tetracycline-inducible cyclohexadienyl dehydrogenase (CDH) which converts prephenate irreversibly into 4-hydroxyphenylpyruvate and eliminates residual growth of *pheA* mutants under acidic conditions (KleeB *et al.*, 2007). While the intracellular growth of the mutant was similar to that of the wild-type strain regardless of the presence or absence of the CDH plasmid (see Fig. 9), further studies were performed with strains harbouring the CDH plasmid.

Intracellular growth of additional mutant strains

To determine whether the absence of a growth defect of the *S. Typhimurium* $\Delta aroP \Delta pheP \Delta tyrP \Delta mtr \Delta pheA$ mutant strain was a trait of the $\Delta pheA$ mutation, we also tested the intracellular growth characteristic of strains that harbor deletions in other genes involved in either aromatic amino acid biosynthesis or uptake, including *tyrA* and *tyrB*, *trpBA*, *pheA* and *trpBA*, *tyrA* and *trpBA*, or *pheA* and *livKHMGF*. The $\Delta livKHMGF$ mutation was included as it has been reported that the branched chain amino acid uptake system in *E. coli* can transport phenylalanine in absence of branch chain amino acids (Koyanagi *et al.*, 2004). As shown in Fig. 10, the intracellular growth of mutants harbouring mutations in one or more different aromatic amino acids are comparable to that of the wild type, indicating that the observed intracellular growth of the *pheA* strain is not specific for that particular strain nor is it specific for a phenylalanine auxotroph, the observation holds true for strains carrying other mutations in the aromatic amino acids. Although the relative growth increases (intracellular CFU at 24 h relative to 4 h post-infection) were similar, for reasons which are not yet clear, the other mutants also showed differing degrees of effects on host cell invasion, likely due to the poor

growth in the bacterial growth media which contains very low levels of tryptophan and tyrosine. We therefore chose to use the $\Delta aroP \Delta pheP \Delta tyrP \Delta mtr \Delta pheA$ mutant strain for the rest of the study.

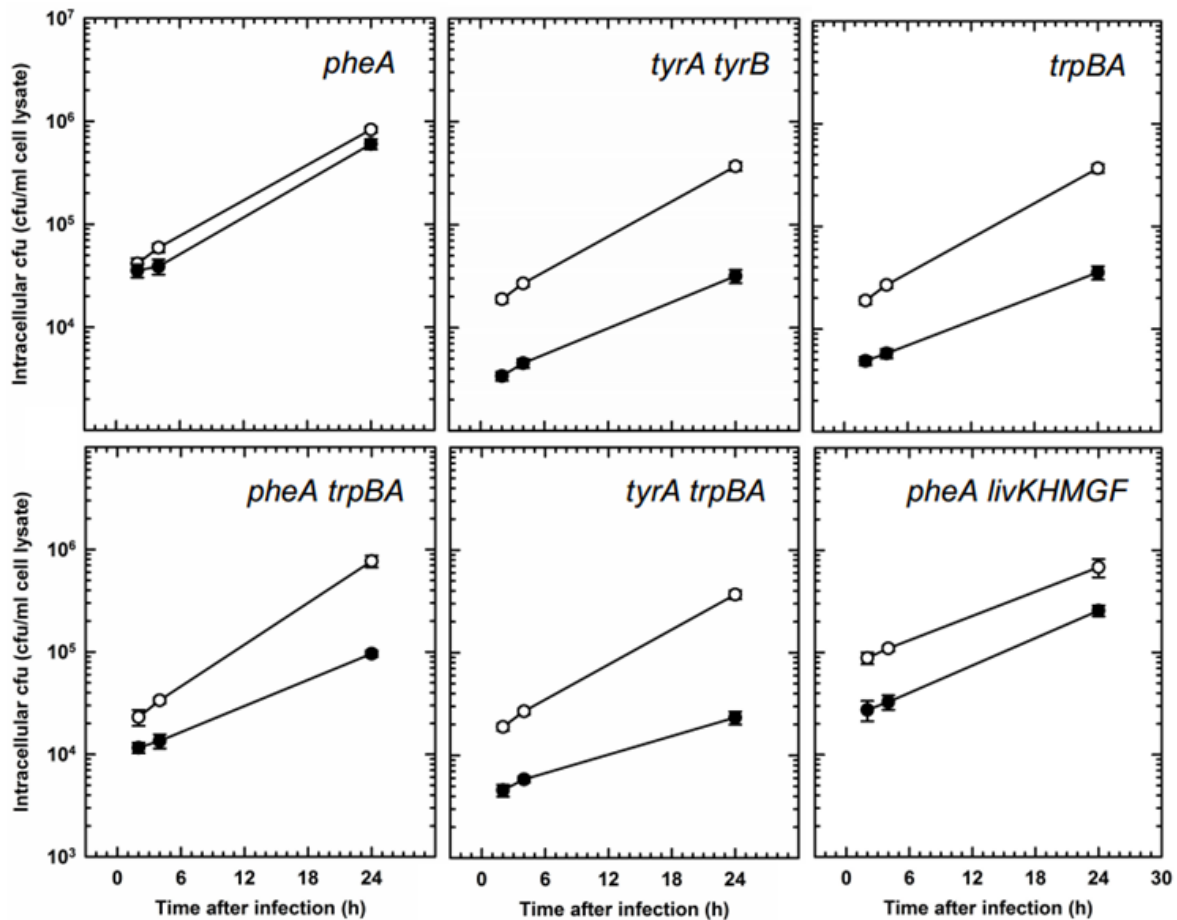


Fig. 10. Intracellular growth characteristics of SL1344 $\Delta aroP \Delta pheP \Delta tyrP \Delta mtr$ strains harbouring additional mutations in aromatic amino acid biosynthetic genes. Shown are the intracellular cfu/ml cell lysate at the indicated time points post-infection in the LoVo human intestinal epithelial cell line for the wild-type (open symbols) and mutant strains (filled symbols) indicated in the panels. The results shown are representative of at least three, independent experiments performed in duplicates.

Intracellular growth in presence of inhibitors against various host cellular mechanism

The observation from the above results that the peptide dependent mutant shows no intracellular growth defect to the wild-type strain, suggests that the growth of mutant is dependent upon an alternative source of phenylalanine other than free amino acids, presumably short oligopeptides. Furthermore, due to the uptake limits of the peptide transporters in *Salmonella*, these peptides should be accessed in partially degraded form

constituting short peptides of about 5 to 6 amino acids to support the growth of the mutant. Also, even if *Salmonella* gets access to host cells oligopeptides, it cannot use them as a source of nutrition as *Salmonella* lacks any extracellular proteases, which further restricts the pathogen to be dependent upon short peptides. We thus considered possible sources of peptides which might be available to intracellular *Salmonella* to contribute to its growth within the SCV.

Every cell (host cell) maintains its cellular homeostasis by targeting proteins, enzymes and other metabolites which are no longer required or whose functions are completed for degradation to lysosomes. As a result, lysosomes generates a rich pool of amino acids and other basic biomolecules which are re-utilized by the host cells for numerous processes. As explained previously, cell could target cargo proteins for degradation to lysosomes via three different forms of autophagy notably, macroautophagy, microautophagy and chaperone mediated autophagy (CMA).

Fusion of the SCV with lysosomal compartment is one such possibility which could provide access to short peptides to the *Salmonella* to support its growth. However, numerous studies over the years have excluded the possibility of a fusion event between lysosomes and the SCV (Garcia-del Portillo and Finlay, 1995; Brumell *et al.*, 2001; Harrison *et al.*, 2004; Brumell and Grinstein, 2004), even though the SCV shares certain host makers common to lysosomes. We thus decided to examine the intracellular growth of the mutant by using inhibitors which block the three forms of autophagy. As depicted in Fig. 11, there is no significant difference in the intracellular growth of the mutant to the wild-type strain upon inhibiting either macroautophagy with 3-methyl adenine or by preventing acidification of lysosomes thus making them functionally inactive using ammonium chloride. Contrastingly, on treating host cells with either cyclohexamide at concentrations inhibiting CMA (Finn *et al.*, 2005) or loading cells with antibodies against Hsc73, which block the activity of lysosomal

form of Hsc73 thus completely blocking CMA without effecting the other forms of autophagy (Agarraberes *et al.*, 1997) resulted in significant growth inhibition of the mutant when compared to the wild-type strain.

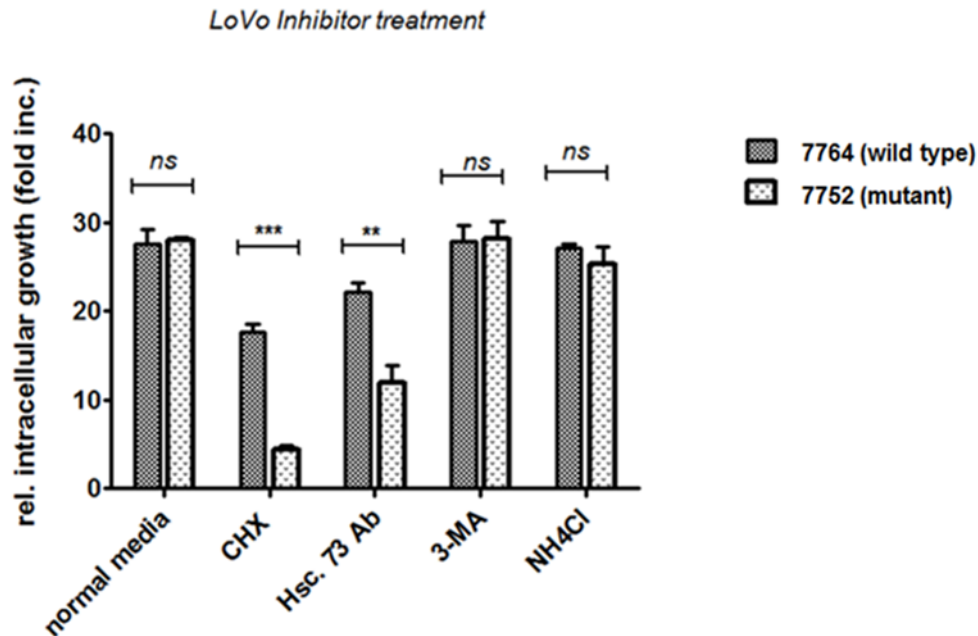


Fig. 11. Blocking chaperone-mediated autophagy inhibits the growth of the peptide-dependent mutant, but not the wild-type strain. LoVo cells were pre-incubated for 4 h with either 200 $\mu\text{g ml}^{-1}$ cyclohexamide (CHX), anti-Hsc73 antibodies, 10 mM 3-methyladenine (3-MA) or 30mM NH_4Cl prior to infection with either wild-type (grey bars) or mutant strains (white bars). The mutant shows reduced growth when CMA is blocked with either CHX or anti-Hsc73 antibodies. Blocking macroautophagy with 3-MA or reducing lysosomal activity by preventing acidification of lysosomes through NH_4Cl , has no effect on the intracellular growth of the mutant strain. The data shown are the means and standard deviations of three, independent assays where, where $p > 0.05$ is considered non-significant (n.s.), **, $p < 0.01$; as determined with Mann-Whitney U-test.

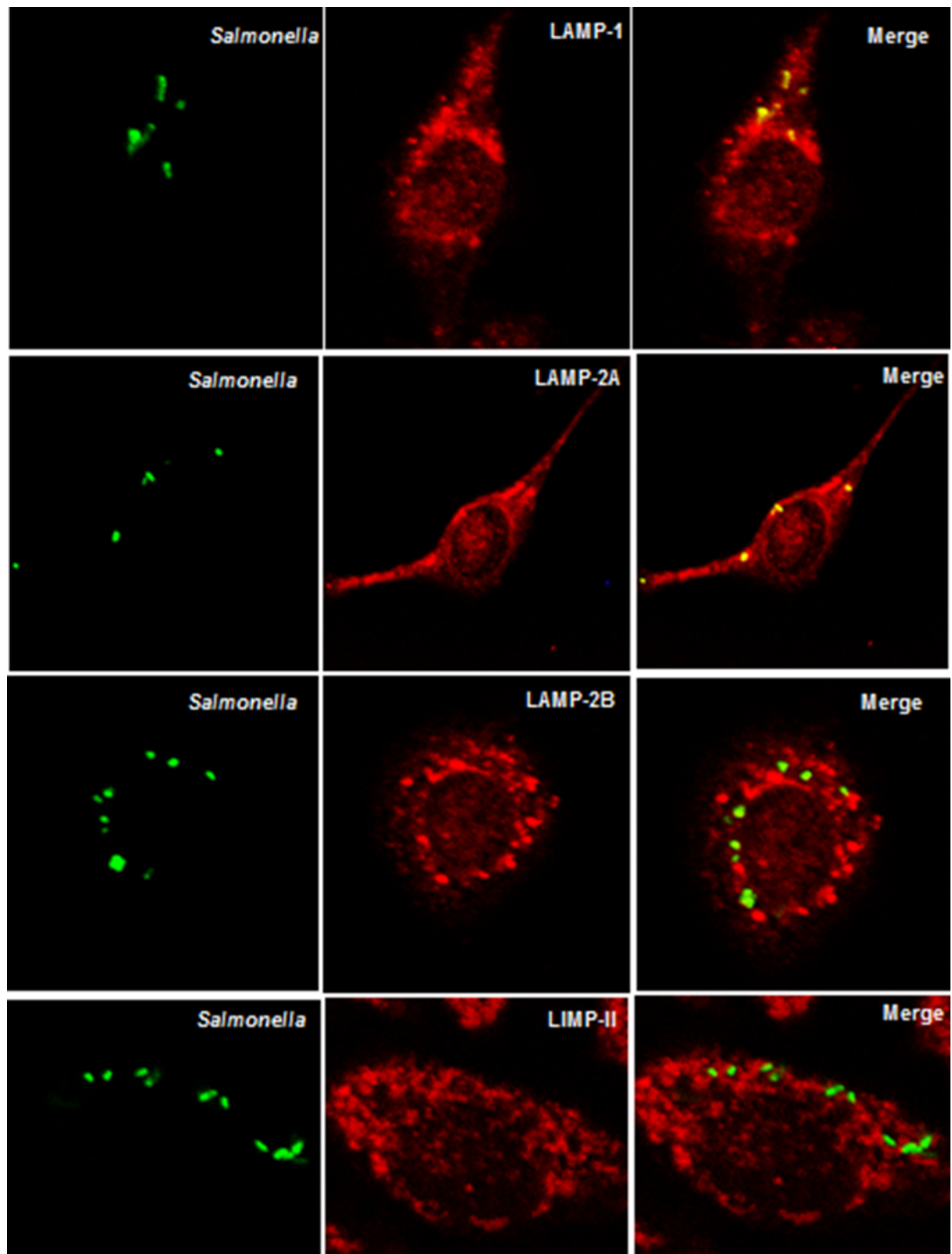
These results suggest that CMA possibly contributes in the intracellular growth of the mutant as similar results were observed in both intestinal epithelial cells and human macrophages. However, at this point in time it still remains unclear on how a pathogen residing inside a vacuole has access to peptides or nutrients destined for CMA. We therefore, decided to examine on the host markers that are present on the SCV membrane.

Co-localization of the *Salmonella* containing-vacuole with components of CMA

Lysosome associated membrane-1 (LAMP-1) and LAMP-2 are well characterized standard markers for the *Salmonella*-containing vacuole (SCV) or phagosome, which are acquired by *Salmonella* on its SCV very early during the invasion process (Steele-Mortimer *et al.*, 1999). Interestingly, there exists three isoforms of LAMP-2, which arises as a result of alternative splicing of the *lamp2* gene and these differ only at the C-terminus region. As described previously only the isoform LAMP-2A is involved in CMA (Cuervo and Dice, 1999), nonetheless, both LAMP-2A and LAMP-2B are found to localize in different membrane micro-domains in the lysosomes (Kaushik *et al.*, 2006).

We were therefore interested to know which isoform(s) of LAMP-2 is associated with the SCV membrane. HEK293T cells were infected with wild-type *Salmonella* expressing GFP at a MOI of 1:5 (cell: bacteria), and 24 h post infection, cells were fixed, permeabilized and incubated with antibodies directed against LAMP-1, LAMP-2A or LAMP-2B. As shown in Fig. 12 (a). As expected, LAMP-1 was found to be associated with the SCV membrane; however, only the isoform LAMP-2A was found to co-localize (yellow appearance around the bacteria) with the SCV membrane, similar to that as observed for LAMP-1. LAMP-2B did not show significant association (co-localization) with the SCV membrane, suggesting that the presence of LAMP-2A on the SCV membrane was not as a result of lysosomal fusion. To further confirm this observation we performed additional co-localization experiments using antibodies against lysosomal integral membrane protein 2 (LIMP-2), the lysosomal receptor for the mannose-6-phosphate-independent transport of β -glucocerebrosidase and other lysosomal hydrolases (Saftig and Klumprman, 2009). As shown in Fig. 12 (a), no significant co-localization with the SCV was observed for LIMP-2. These results further supported the suggestion that fusion with lysosomal compartments was unlikely to serve as a source of peptides for the mutant.

a)



b)

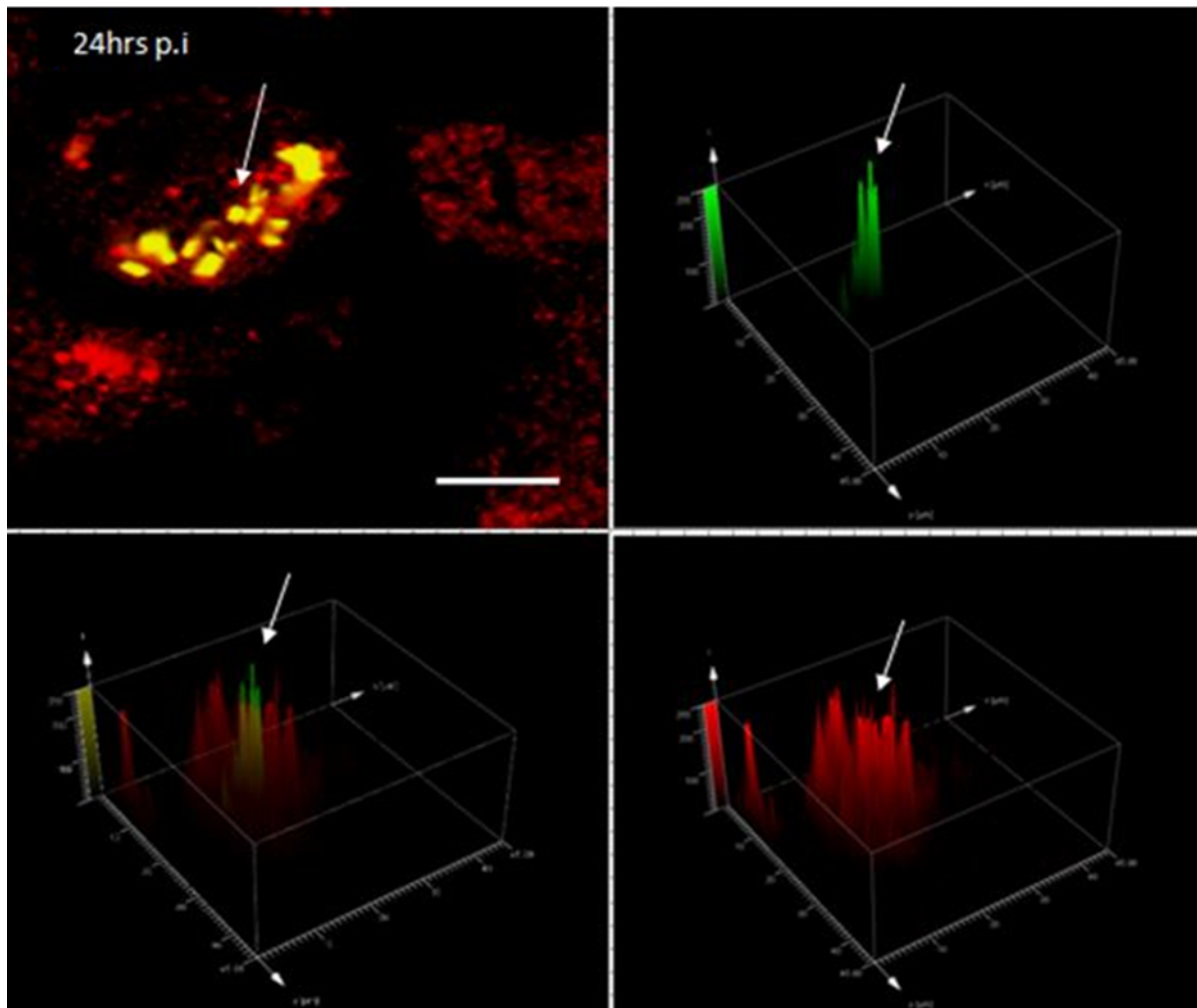


Fig. 12. Co-localization of key components of host chaperone mediated autophagy with the *Salmonella*-containing vacuole. Wild-type *Salmonella* expressing GFP (green) were used to infect HEK293T cells grown on coverslips at a multiplicity of infection (MOI) of 5. 24 h post-infection, cells were washed with PBS, fixed, and permeabilised with 0.1% Triton X-100. After blocking, the infected cells were incubated overnight at 4°C with the indicated primary antibodies. The following day, the coverslips were washed, and incubated with the appropriate secondary antibodies conjugated to Alexa-Fluor 546 (red) and examined by laser scanning confocal microscopy. (b) Co-localization of *Salmonella* with Hsc73, co-localization (yellow) was confirmed with heat intensity plots. The images shown are representative of at least 25 individual cells per condition performed in duplicate on at least three, independent occasions.

As LAMP-2A was found to co-localize with the SCV, we next determined whether the other major component of the CMA complex, Hsc73, was also associated with the SCV membrane. As shown in Fig. 12 (b), antibodies specific for Hsc73 were also found to co-localize with the SCV of intracellular *Salmonella*. Similar results were obtained in intestinal

epithelial cells and human macrophages, suggesting that the association with CMA components is not a cell type effect.

In addition we also, quantified the percentage of intracellular bacteria which were associated with these host cell components. As can be seen in Fig. 13, nearly, 80- 85% of total intracellular bacteria harbors LAMP-1, LAMP-2A and Hsc73 on the SCV membrane, whereas the other lysosomal markers such as LAMP-2B, LIMP-2 and M6PO4 (Mannose 6 Phosphate) receptor were absent from the SCV membrane.

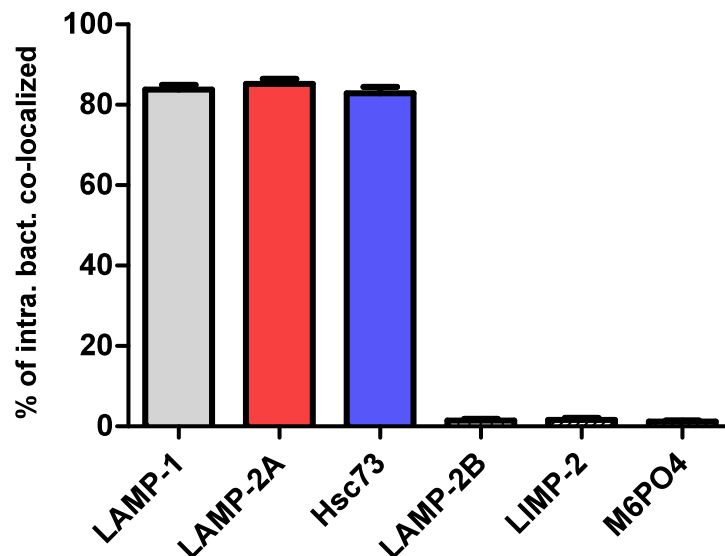


Fig. 13. Quantification of percentage of intracellular bacteria localized with various markers in the host. Of the total intracellular bacteria about 80-85% were found to be associated with LAMP-1, LAMP-2A and Hsc73, whereas there was no significant association with other lysosomal markers namely, LAMP-2B, LIMP-2 and M6PR receptor.

Furthermore, we also performed kinetic studies in order to determine, whether LAMP-2A is associated during the early phase of infection around the SCV membrane. As shown in Fig. 14, the presence of LAMP-2A can be seen as early as 1 h post infection and its localization around the bacteria increases with time. Although the presence of LAMP-2A at an early time point does not necessarily imply that the CMA apparatus is functionally active and is being

utilized by *Salmonella* as a source for nutrition. But at later time points at about 20-24 h post infection, the acquisition of chaperone Hsc73 and Lamp-2A confirms functional activity of CMA to support bacterial growth.

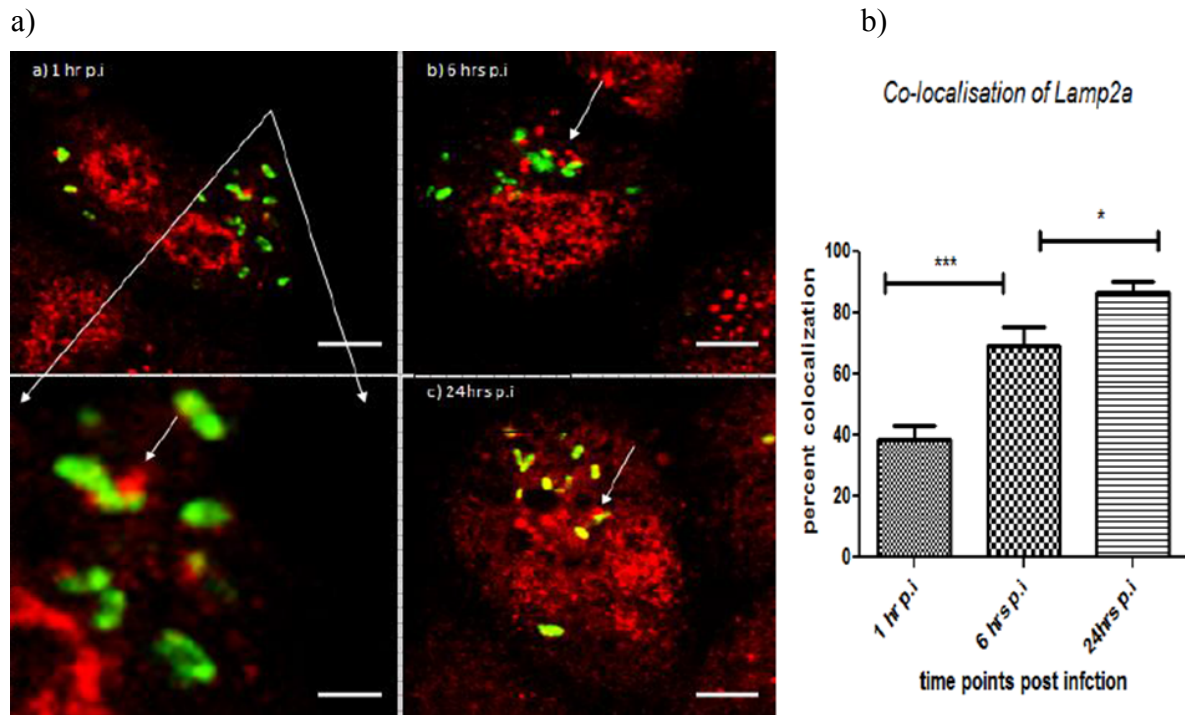


Fig. 14. Kinetics of LAMP-2A co-localization. (a) HEK293T cells were infected with wild type GFP expressing *Salmonella* (green). At the indicated time points p.i the monolayer's were fixed and immuno-stained with anti-LAMP-2A (red). Localization of LAMP-2A around the bacteria is indicated with arrow heads (white) and percent co-localization was quantified at different time point's p.i. (***= p value < 0.01, *=p<0.5 indicates the significance as obtained using Mann Whitney student t test, scale bar= 8μm)

Effect of LAMP-2A and LAMP-1 knock down on intracellular growth.

As shown above that both LAMP-1 and LAMP-2A localizes with *Salmonella*-containing vacuole, and only LAMP-2A have been reported to have a function in CMA, we therefore examined the intracellular growth of the mutant and the wild type strain in LAMP-1 and LAMP-2A knockdown cells. We performed shRNA mediated stable knock-down of LAMP-2A and LAMP-1 in HEK293T cells and compared the intracellular growth of the mutant and wild-type strains.

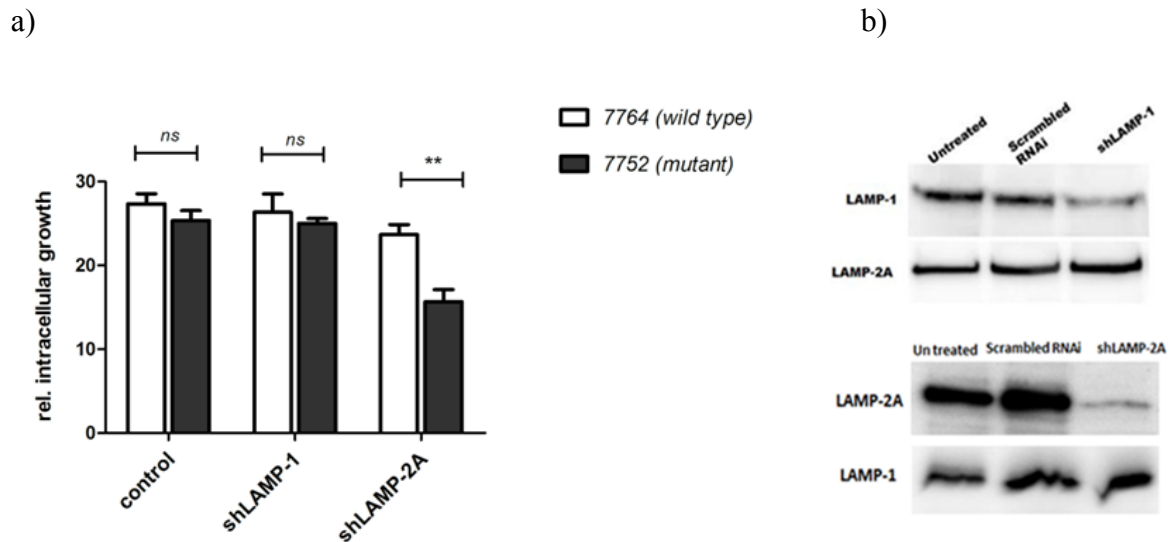


Fig. 15. Intracellular growth of mutant is effected by LAMP-2A knockdown. The intracellular growth of peptide dependent mutant is significantly reduced in LAMP-2A knockdown cells, however LAMP-1 knockdown does not affect the growth of mutant. A) Confirms knockdown of LAMP-1 and LAMP-2A as verified by western blots of the whole cell lysates. b) Shows the relative intracellular growth of wild type (clear bar) and peptide dependent mutant (black bars) in control, LAMP-1 and LAMP-2A knockdown HEK293T cells. The data shown are the means and standard deviations of three, independent assays where , where $p > 0.05$ is considered non-significant (n.s.), **, $p < 0.01$; as determined with Mann-Whitney U-test.

As shown in Fig. 15, reduced expression of LAMP-2A significantly inhibited the growth of the peptide-dependent mutant strain compared to the wild-type, further supporting an involvement of CMA in intracellular growth of the mutant. However, LAMP-1 knockdown did not have any effect on the intracellular growth of both wild-type and mutant strain. Significant reductions in the levels of LAMP-2A and LAMP-1 were verified in Western blots of whole cell lysates of transfected cells. These results indicated that not only were key components of the CMA complex present at the SCV membrane, but functionally active CMA was also required to support intracellular growth of the peptide-dependent strain.

Co-localization of the CMA substrate (GAPDH) with the *Salmonella*-containing vacuole

Both the co-localization and inhibitor studies strongly suggested that the host CMA components are associated with the SCV and thus CMA could possibly contribute to the intracellular growth of *Salmonella* within the SCV, however it is still not clear, whether the CMA apparatus is functionally active at the SCV membrane. We were thus interested in

verifying whether a cytosolic CMA substrate translocates to the SCV. For this purpose we used one of the best characterized CMA substrate GAPDH (Aniento *et al.*, 1993; Cuervo *et al.*, 1994). We therefore transfected HEK293T epithelial cells with the vector pCMV-GAPDH-HT expressing a HaloTag fusion with GAPDH which binds fluorescent ligands under neutral (cytosolic), but not acidic (lysosomal) conditions.

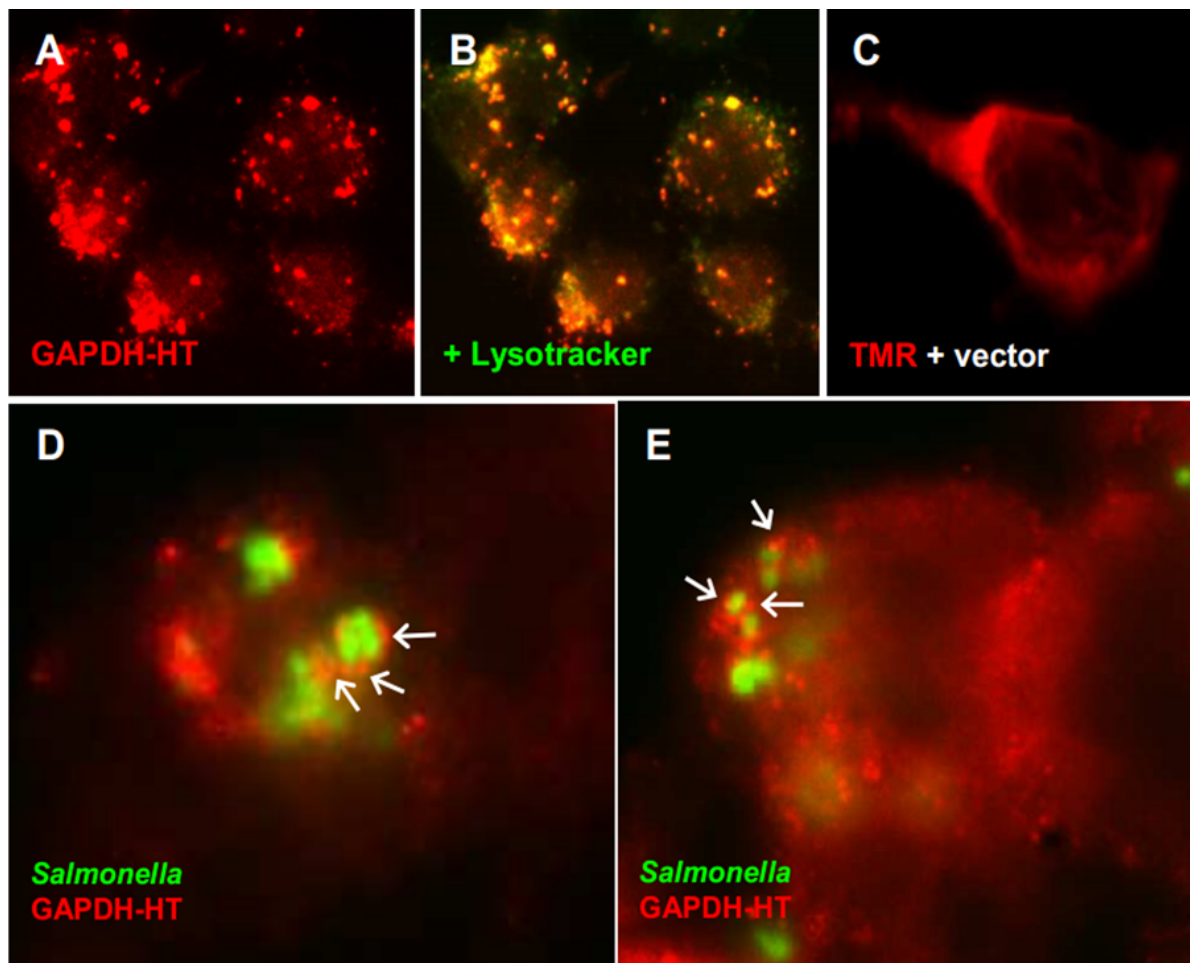


Fig. 16. Co-localisation of the CMA substrate GAPDH with the *Salmonella*-containing vacuole. HEK293T cells stably transfected with the pCMV-GAPDH-HT plasmid, were incubated for 4 h with 500 nM TMR-HT ligand prior to infection with *S. Typhimurium*. 24 h post-infection, cells were fixed and visualized by laser confocal scanning microscopy. Shown are the punctate patterns of TMR-HT ligand (A) and co-localisation with lysosomal compartments stained with LysoTracker Green (B). (C) Control transfections with pCMV vector stained with the TMR-HT ligand. Representative pictures of co-localisation of GFP-expressing *Salmonella* with GAPDH-HT (D, E). The pictures shown are representative of images from two, independent experiments in duplicate and at least 25 fields of visualization.

On incubating the cells with TMR ligand, it binds with the HaloTag in the cytosol, the GAPDH-HT: ligand complex continues to fluoresce and can therefore be used to study translocation of CMA substrates such as GAPDH into lysosomes (Seki *et al.*, 2012). The transfected cell line was then infected with GFP expressing *Salmonella* for co-localization studies. As shown in Fig. 16, after incubating the transfected cells with the fluorescent ligand TMR, co-localization of red fluorescent, punctate complexes were found in close proximity to the SCV. These results were therefore suggested that not only the components of CMA are associated with the SCV but also CMA is functionally active at the SCV membrane as we successfully showed transport of at least one genuine CMA substrate to the SCV.

Co-localization with plasmid expressing KFERQ-DsRed target motif with SCV

As 30% of host cytosolic proteins bearing the KFERQ amino acid motif in their protein sequence are target to lysosomes by Hsc73 and eventually degraded via CMA, ubiquitously in all cell types. In-order to further confirm our previous observation that CMA is functionally active at the SCV membrane as GAPDH-HT:ligand complex were present in close proximity of SCV we therefore transfected HEK293T cells with a vector containing KFERQ sequence fused with DsRed. Similar to other CMA substrates, the KFERQ-DsRed fusion transcript should be targeted to lysosomes or vesicles containing CMA components for degradation. The transfected cell line was subsequently infected with GFP expressing *Salmonella*. 20 h post infection, the cells were fixed and subsequently imaged. As shown in Fig. 17, the KFERQ-DsRed transcript product in transfected cells co-localized with GFP-expressing *Salmonella*.

The co-localization further confirms our previous results that not only the CMA components, LAMP-2A and Hsc73 associated with the SCV, but further that CMA is functionally active at the *Salmonella*-containing vacuolar membrane. Furthermore, in addition to GAPDH, the localization with KFERQ-DsRed fusion product indicates, that the substrate proteins presumably of CMA are targeted to SCV, which could possibly serve as source of nutrients for the bacteria residing within the membrane bound compartment, the SCV.

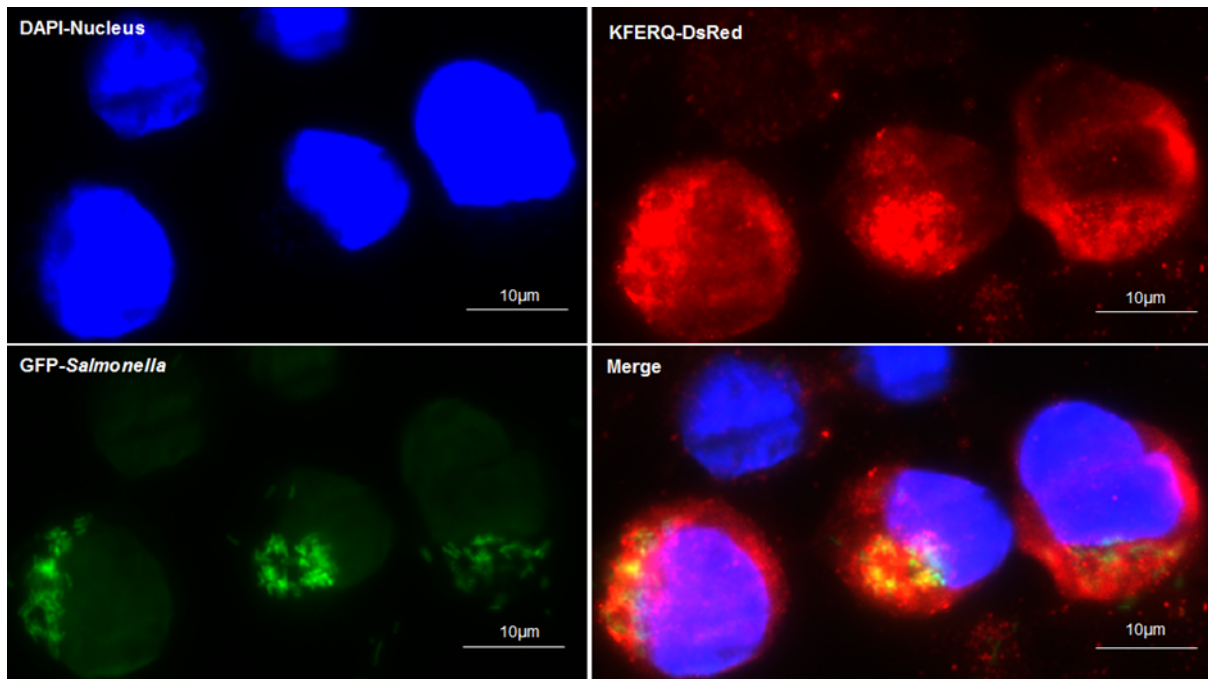


Fig. 17. Co-localisation of the KFERQ-DsRed fusion product with the *Salmonella*-containing vacuole. HEK293T cells were transfected with pCDNA3.1 –KFERQ-DsRed plasmid and selected using neomycin. The transfected cells were then infected with GFP expressing *Salmonella*, after 20hrs p.i the cells were fixed and imaged. KFERQ-DsRed construct were found to be co-localized with GFP-*Salmonella*.

Isolation of *Salmonella*-containing vacuole using magnetic nanoparticles

A major problem in determination of interacting host proteins and compartments with the pathogen containing phagosome has been the isolation of phagosomes free of spurious or contaminating proteins and vesicles. Buoyant density gradient centrifugation has generally been applied, in which latex beads are pre- or post-loaded with phagosomes containing pathogens and purified from fractions taken from the gradients where the latex particles show a discrete banding pattern (Desjardins *et al.*, 1994). However, this method is dependent on separation from within a gradient of vesicles with similar densities, and cross-contamination is unavoidable. Magnetic Cell Separation (MACS) and other column-based methods require removal of non-bound material with extensive washing steps, and labile compartments such as phagosomes may lose considerable amounts of material and loosely bound proteins. An improvement has been the application of Flow-Assisted Cell Sorting (FACS), in which GFP- or other fluorescently-labeled bacteria are used to infect cells, and after lysis of the cells, the cellular contents are passed through a flow cytometer and fluorescently labelled components are diverted from the flow-through for collection. However, the shear forces generated can lead to disruption of the phagosomes and the volumes required lead to inefficient concentration of the samples.

We therefore developed a method of SCV isolation which was rapid and required a minimum of manipulations in contrast to column- or flow-assisted cell sorting (FACS)-based methods where shear forces might lead to disruption of the labile SCV membrane. Furthermore, our main objective was to avoid any form of covalent labeling of the bacterial outer membrane to ligands with chemical reagents (Lönnbro *et al.*, 2008) which might affect the function of the type III secretion apparatus needed for secretion of virulence proteins necessary for establishment of the intracellular niche. We therefore labeled *Salmonella* with carbon-coated, paramagnetic cobalt nanoparticles prior to infection in the host cells, followed by recovery of the bacteria after lysis of host cells using a magnetic field.

Attachment of carboxyl coated magnetic nanoparticles on the bacterial surface

Bacterial attachment to any surface is related to surface charges both on the bacteria as well as on the substratum or target of attachment. Dickson *et al.*, 1989 characterized surface charges and their co-relation to bacteria attachment. As per the study, *Salmonella typhimurium* was found to consist of an overall net negative charge with r/e (-) 9.47 to r/e (+) 4.78. We therefore, utilized this small, though significant positive charge on to the bacterial surface to electrostatically label the bacteria with paramagnetic nanoparticles. For this purpose, we used, paramagnetic cobalt nanoparticles of diameter 10 - 50 nm; (Turbo Beads, Zurich) with a carboxyl functional group attached to their surface, thus providing the net negative charge for attachment via electrostatic interactions on to the positive charge areas (surface) of *Salmonella*. Another, reason for selecting to target the positive charge of *Salmonella* in contrast to the majority negative charge, was, we were not certain about the effect of binding of these particles on to the behavior of the bacterium, as otherwise it would have led to more particle binding to bacteria and it could have effected, the SPI-I or SPI-II functionality, and eventually the isolated SCV, would no longer be a representation of *Salmonella's* natural cell trafficking within the cell.

Sonicated, centrifuged nanoparticles were thus incubated with bacterial pellets resuspended in 1X PBS, at a ratio of 5:1 ratio of nanoparticles: bacteria at 37°C for 20 min. with shaking. The bacterial suspension was then filtered through a 0.2 µm filter fitted to a 5 ml syringe. The filters were washed twice with two volumes of PBS so as to get rid of any un-bound or free floating nanoparticles, the filter was then inverted and the bacteria were recovered in 1 ml of PBS collected as a result of backwash into a sterile microfuge tube. These recovered bacteria were then further utilized either for infecting THP-1 human macrophages or processed for transmission electron microscopy. As shown in Fig. 18 (a) & (b), the bacteria collected after the backwash, clearly depicted association with magnetic nanoparticles; furthermore the attachment is not uniformly distributed over the entire bacterial surface, but was only

concentrated at specific points in aggregation. Additionally, intracellular *Salmonella* retained the electrostatically attached magnetic nanoparticles, within human macrophages as shown in Fig. 18 (d), here the nanoparticle tagged bacterium can be seen inside a vacuolar vesicle, presumably the SCV or an endosomal compartment.

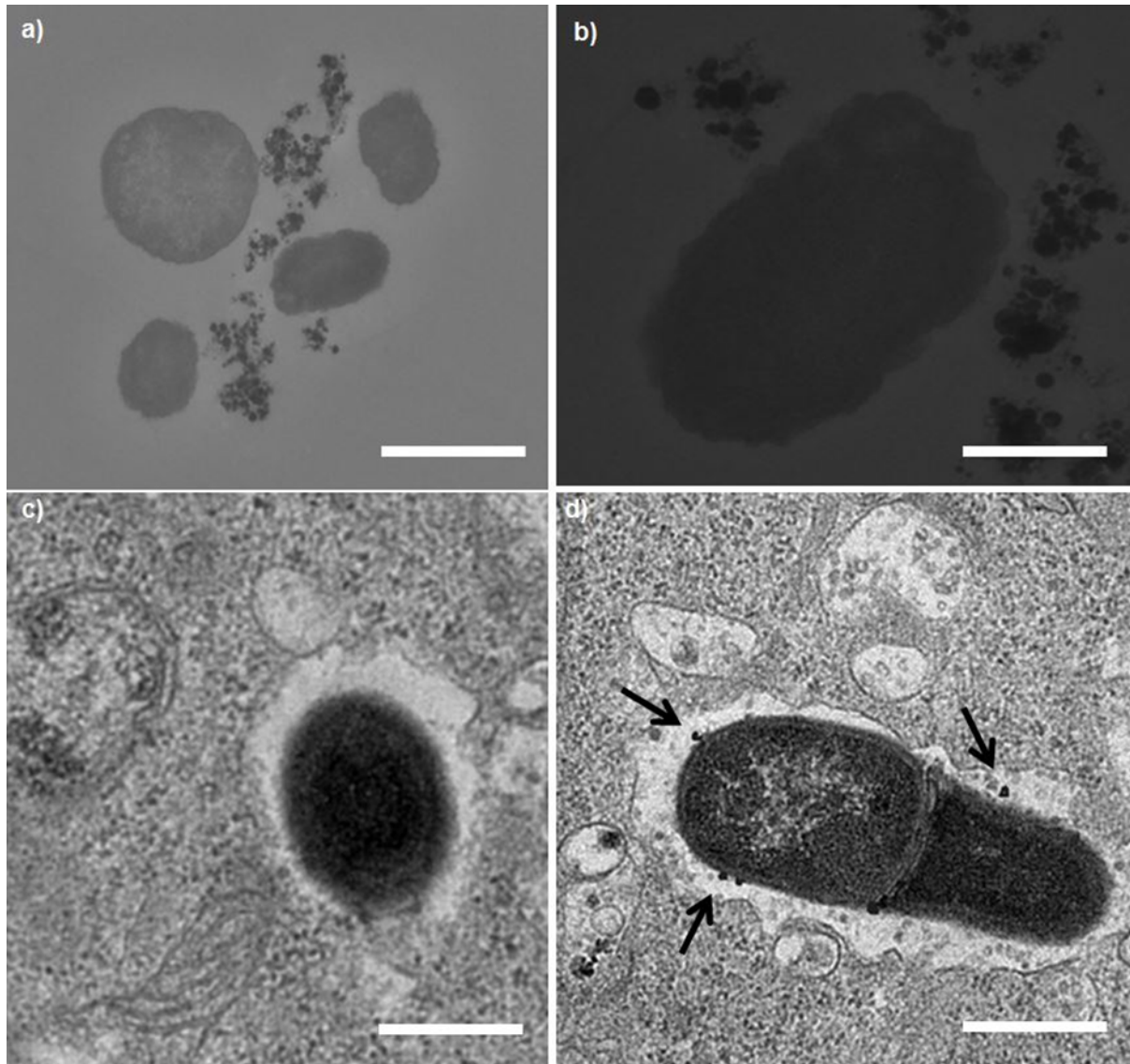


Fig. 18. Attachment of carboxyl coated magnetic nanoparticles on to the bacterial surface. (a) (b) represents TEM images of magnetic nanoparticles, attached to bacteria surface marked with arrow heads. (c) & (d), represents intracellular *Salmonella* in THP-1, human macrophage cell line untagged (control) and tagged with nanoparticles as marked with arrow heads respectively. Scale bar 1 μm.

No effect on bacterial viability and invasion rates of nanoparticle-labelled bacteria.

In order to be certain, that the attachment or interaction of *Salmonella*, with paramagnetic nanoparticles had no significant effect on the viability of the bacteria. We therefore, examined the c.f.u of *Salmonella* before and after incubation with magnetic nanoparticles, under shaking conditions at 37°C. Sonicated, nanoparticles were incubated, with bacterial pellets resuspended in 1X PBS, at a ratio of 5:1 & 10:1 ratio of nanoparticles: bacteria at 37°C for 20 min. and 40 min respectively under continuous shaking conditions. The c.f.u was plated before and after incubation, in order to determine the effect (if any) of nanoparticle attachment on the viability of *Salmonella*. As shown in figure 19 (b), the total c.f.u before incubation and after incubation with magnetic nanoparticles, both at a higher ratio and for longer time period of incubation, remains same, suggesting that the nanoparticle did not affect the viability of the bacteria.

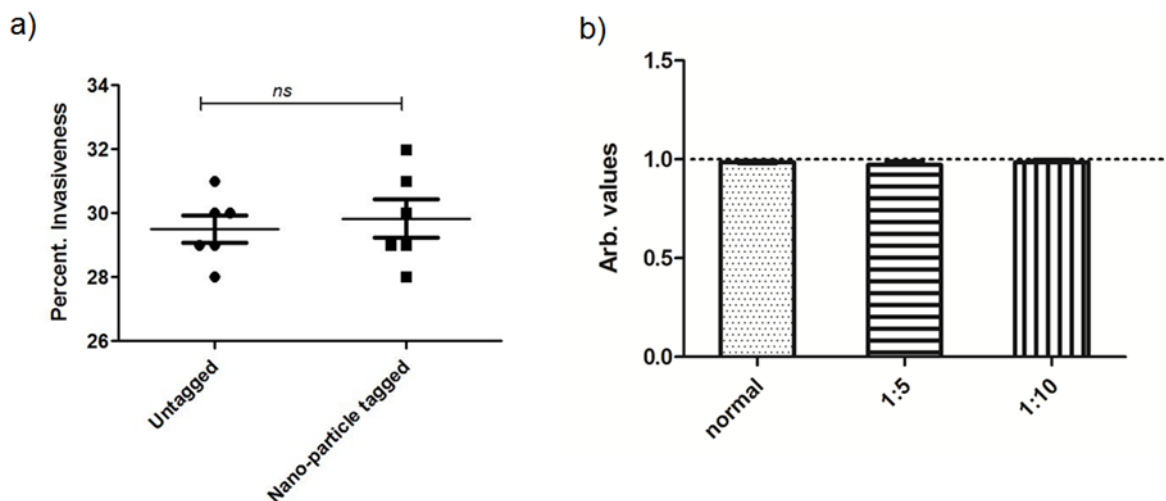


Fig. 19. Effect on Invasion rate and viability of bacteria upon attachment with nanoparticles. a) Confluent monolayers of THP-1 macrophages were infected with nanoparticle labeled and non-labelled bacteria at an MOI of 1:5 (cell: bacteria) to compare the invasion rates after 2 h p.i. b) Bacteria were incubated with nanoparticles for 20 min and 40 min, and viability was determined by determining the intracellular the cfu/ml cell lysate.

We then further determined, whether the attachment of nanoparticles had any effect on the invasion process of *Salmonella*. For this purpose, we infected pre-seeded THP-1 human macrophages, with untagged *Salmonella* or nanoparticle tagged bacteria at a MOI of 1:5 (cell: bacteria). Invasion rates were determined by dividing the intracellular c.f.u at 4 h p.i to that of 2 h p.i both for control group and the test group. As depicted in Fig. 19 a) the labeling of bacteria with paramagnetic nanoparticles had no significant effect on the invasion rates of *Salmonella* as both the bacterial groups were found to be 30% invasive in THP-1 cells.

SCV purification and analysis

Salmonella expressing green fluorescent protein (GFP) and bound with carbon-coated, paramagnetic cobalt nanoparticles, were used for infecting THP-1 macrophage cells at an MOI of 1:10 (cell: bacteria). After 24h post infection, the host cells were lysed and bacteria were recovered in the presence of a magnetic field. The recovered cell lysates were then placed in a magnetic field (Single Place Magnetic Stand, Ambion, USA) and the supernatant was carefully removed and discarded, and replaced by 1 ml of a 1X PBS/2% sucrose solution. The recovered bacteria were then further processed for analysis either by microscopy or Western blotting, by examining the presence or absence of the SCV markers at the membrane surface.

a) Microscopic analysis

Purified SCV's were incubated in the presence of antibodies against LAMP-1 and examined by fluorescence microscopy. As shown in Fig. 15 (upper row), GFP-expressing *Salmonella* showed exterior labeling with LAMP-1, indicating that the SCV membrane in these preparations was intact. Also, as shown previously in Fig 12, that intracellular *Salmonella* within the SCV is associated with LAMP-2A, therefore we also incubated the purified SCV's in presence of antibodies against LAMP-2A. Consistent with our previous results, the purified SCV also show presence of LAMP-2A as shown in Fig. 20 (bottom row) on its membrane surface, similar to that as observed for LAMP-1.

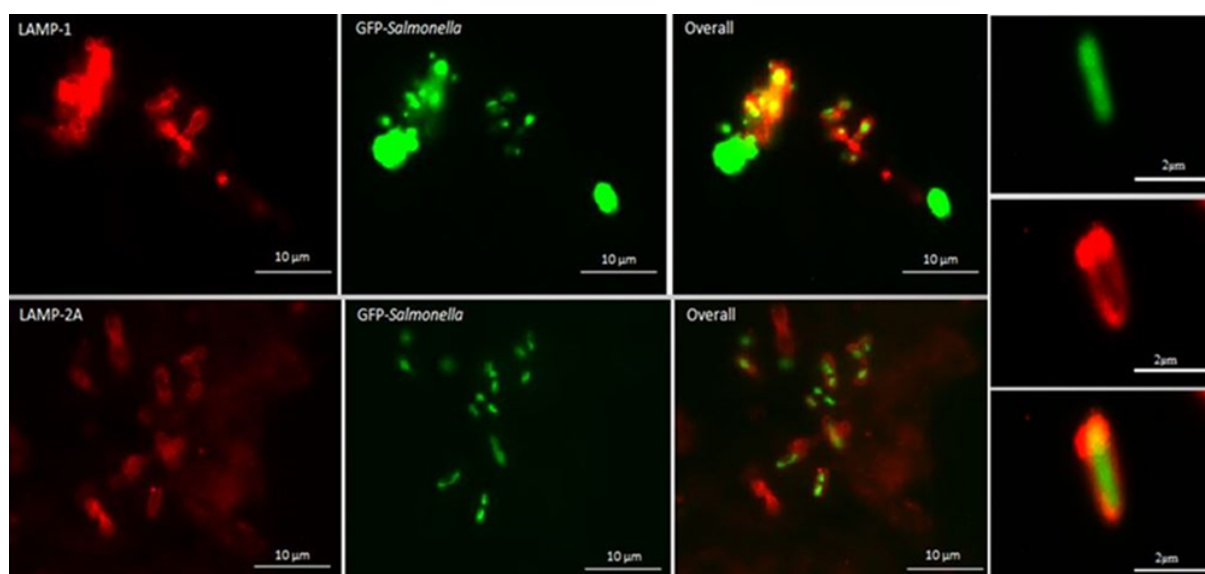


Fig. 20. Microscopic analysis of purified SCV. Representative immunofluorescence pictures of purified SCVs recovered from THP-1 macrophage infected with paramagnetic nanoparticle labeled, GFP-expressing *Salmonella* and stained for LAMP-1 (upper row). The lower row shows acquisition of LAMP-2A on the intact membrane of the SCV. The right panel is a close-up of an individual SCV showing a single bacterium (left), LAMP-1 staining (middle) and a merge of the two emission spectra (right).

These results indicated that the isolated SCV's contained both LAMP-1 as well as LAMP-2A. Moreover, the labelling with both LAMP-1 and LAMP-2A at the SCV membrane also indicated that the membrane of these isolated SCV's is intact. Furthermore, these results with isolated and purified SCV's further confirmed our previous observation that the key CMA component LAMP-2A is associated with *Salmonella*-containing vacuole (SCV).

b) Western blot analysis

In order to further verify the presence or absence of SCV-associated host proteins on the SCV membrane, we performed Western blotting with the purified *Salmonella*-containing vacuoles.

Salmonella expressing green fluorescent protein (GFP) were bound with carbon-coated, paramagnetic cobalt nanoparticles prior to infection of macrophage, followed by recovery of the bacteria after lysis of host cells using a magnetic field. As a control, nanoparticle-bound *Salmonella* were heat-killed, and incubated with macrophage for uptake by phagocytosis.

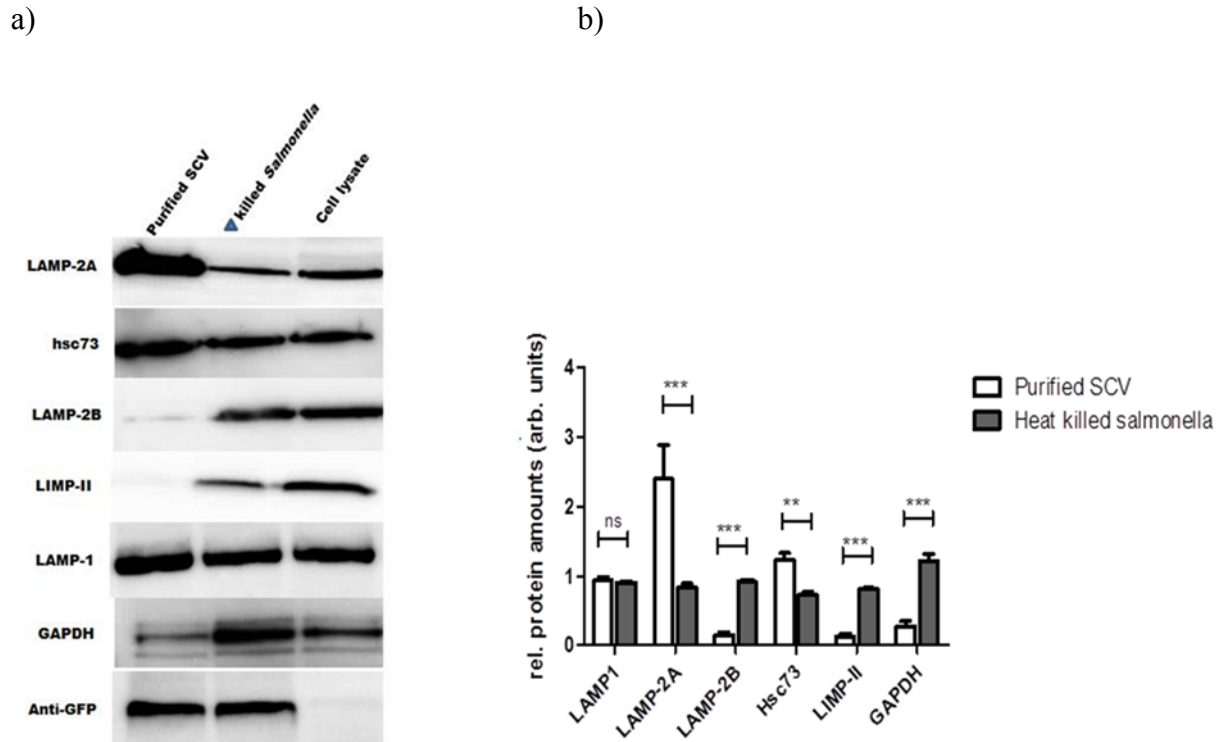


Fig 21. Western blot analysis of purified SCV. Purified SCVs recovered from THP-1 macrophage infected with either live (SCV) or heat-killed (killed) GFP-expressing *Salmonella* were subjected to SDS-PAGE and Western blotting using antibodies against the indicated host proteins. **(b)** Quantification of the relative amounts of the indicated host proteins present in SCVs purified from macrophage infected with either live (open bars) or heat-killed *Salmonella*. Signal intensities were determined relative to a constant amount of non-infected cell lysates. The data shown are the means and standard error of at least two, independent experiments. Statistical significance was determined by a two-way Anova, where $p > 0.5$ is considered non-significant (n.s.); **, $p < 0.01$; ***, $p < 0.001$.

The purified SCV preparations were then subjected to SDS-PAGE followed by Western blotting using specific antibodies against various lysosomal markers. As expected, LAMP-1, LAMP-2A and Hsc73 were present in purified SCV preparations, whereas LAMP-2B and LIMP-2 were significantly reduced or absent compared to control phagosomes containing heat-killed *Salmonella*, which showed the prominent presence of lysosomal markers, as expected for mature lysosomes (Fig. 20a). Interestingly, we observed significantly higher amounts of both LAMP-2A and Hsc73 in SCV preparations with live *Salmonella* compared to phagocytosed dead *Salmonella*, suggesting an enrichment of these proteins at the SCV membrane as demonstrated in the quantification plot shown in Fig. 20 (b).

Furthermore, the CMA substrate, GAPDH was also present in low, but appreciable levels in the SCV preparations. These results further confirmed our previous observation as shown in Fig. 16 with co-localization studies using a pCMV-GAPDH-HT construct where SCV co-localized with GAPDH (CMA substrate), indicating functional nature of CMA on SCV membrane.

Discussion

Autophagy: a friend or foe for *Salmonella*?

Autophagy is a fundamental catabolic process of eukaryotic cells which is required for the elimination of cytosolic protein aggregates, denatured or damaged proteins, enzymes which are no longer required, and damaged cytosolic organelles by delivering them to lysosomes for degradation. In this manner, autophagy plays an essential role in maintaining cellular homeostasis in response to numerous cellular and environmental stresses. Autophagy pathways also play an important role in multiple aspects of innate and adaptive immunity (reviewed by Virgin and Levine, 2009; Levine *et al.*, 2011).

Autophagy also plays a crucial role in generating innate immune responses against microbial infections. During infections, autophagy is responsible for the recognition, capture, selective targeting and degradation of intracellular bacteria and viruses (Levine, 2005; Deretic, 2011). Antibacterial autophagy also plays a role in controlling intracellular bacterial replication and generating host innate immune responses in host cells. Evidence from recent studies has suggested that intracellular bacteria residing within vacuoles can be targeted to lysosomes for degradation by activation of autophagy (Levine, 2005; Deretic, 2011). Furthermore, access to the cytosol for intracellular bacteria, caused by damaged vacuoles, allows autophagic targeting of bacteria by delivering them to lysosomes (Ogawa *et al.*, 2009; Collins and Brown, 2010; Fujita and Yoshimori, 2011).

Autophagosomes, in addition to sequestering cytosolic material for degradations also play a critical role in selectively targeting material derived from intracellular pathogens for degradation by means of receptor-ligand interactions. Intracellular bacteria can induce the formation of ubiquitinated protein aggregates which are recognized by cargo adaptors or receptors meant to target these for degradation (Collins and Brown, 2010; Fujita and Yoshimori, 2011). Well-characterized autophagy receptors such as p62 (sequestosome 1 or SQSTM1) (Pankiv, *et al.*, 2007), NBR1 (neighbour of BRCA1 gene 1) (Kirkin *et al.*, 2009),

NDP52 (nuclear dot protein, 52 kDa) (Thurston *et al.*, 2009) and OPTN (optineurin) (Wild *et al.*, 2011) together represent a category of pattern recognition receptors, known as sequestosome 1/p62-like receptors (SLRs) and these link autophagy to innate immunity (Deretic *et al.*, 2011 and Ogawa *et al.*, 2011). These receptors bind to both ubiquitin and ATG8 family of proteins such as LC3s and GABARAPs and eventually mediate targeting of ubiquitinated cargo to autophagy (Johansen and Lamark, 2011).

Salmonella serovar *typhimurium* is a facultative intracellular pathogen which infects a wide variety of hosts. *Salmonella* follows a bimodal life style within its host cells. The pathogen usually resides in a vacuolar compartment known as the *Salmonella*-containing vacuole (SCV), within which the pathogen replicates and secretes a number of effector proteins through type III secretion systems into the host cell cytosol. Studies have found that the bacterial type III secretion system, which spans both the bacterial and SCV membranes, can lead to damage of the SCV membrane leading to bacterial escape into the host cell cytosol where they can be detected by the autophagy process (Birmingham *et al.*, 2006). In the HeLa cell line, approximately, 25% of *Salmonella* Typhimurium become cytosolic, where the bacteria become decorated with polyubiquitinated proteins, and the ubiquitinated bacteria are then detected by cargo adaptor, NDP52 (Thurston *et al.*, 2009). *Salmonella* Typhimurium invasion of host cells also leads to activation of the TLR4 signalling pathway, which leads to phosphorylation of TBK-1 (Yuk and Jo, 2011). Through a cascade of signalling events, this in turn leads to recruitment of NDP52, which then results into phosphorylation of OPTN, another autophagy receptor (Thurston *et al.*, 2009; Wild *et al.*, 2011). Phosphorylation of OPTN further enhances its ability to interact with autophagic proteins LC3, enabling the elimination of the pathogen by activation of a form of autophagy known as xenophagy (Wild *et al.*, 2011). Furthermore, recent studies have revealed that NDP52 preferentially interacts with the LC3 isoform C (LC3C) and this selective interaction is involved in recruitment of

ATG8 protein family proteins to cytosolic bacteria which can result in elimination of *Salmonella* Typhimurium (von Muhlinen *et al.*, 2012).

Diacylglycerol (DAG), a lipid second messenger generated by phospholipase D, has also been reported to be associated with autophagy-targeted *Salmonella*, and is essential for antibacterial autophagy through protein kinase C δ signalling (Shahnazari *et al.*, 2010). Additionally, a recent study has revealed that the cytosolic lectin Galectin 8 (LGALS8) plays a novel role in detecting bacterial invasion by binding to host glycans during invasion by *Salmonella*. This binding of Galectin 8 leads to NDP52 recruitment eventually resulting in antibacterial autophagy (Thurston *et al.*, 2012). A similar function for LGALS8 has been reported in case of *Shigella* invasion.

Another cargo adaptor, p62/SQSTM1, is recruited by polyubiquitin-coated *Salmonella* for degradation of the pathogen by xenophagy (Zheng *et al.*, 2009). NBR1 is another cargo adaptor which possesses a similar domain structure required for interaction with LC3 proteins and ubiquitin. The N-terminal region of NBR1 contains a PBI domain which is required for interacting with LC3 proteins, and its C-terminal regions possess a UBA domain which interacts with ubiquitin (Kirkin *et al.*, 2009; Lamark *et al.*, 2009). NBR1 interacts with p62 to form oligomers which are recruited to polyubiquitinated *Salmonella* which can then be degraded by autophagy processes (Kirkin *et al.*, 2009; Lamark *et al.*, 2009).

Salmonella targeted by autophagy are either labelled with ubiquitin or DAG, suggesting that at least two independent pathways promote bacterial degradation. Furthermore, recruitment of NDP52 by Galectin 8 to damaged vacuoles suggests involvement of non-ubiquitin pathways also involved in targeting bacteria for clearance through autophagy. In fact, Galectin 8-mediated recruitment of NDP52 shows a temporal pattern of autophagy induction, *i.e.* Galectin 8-mediated recruitment of NDP52 occurs first, followed by ubiquitin-dependent NDP52 recruitment. These observations highlight that different pathways

can act at distinct steps during bacterial infection and results in targeting and clearance of the pathogen by autophagy.

In contrast, a recent study examined the fate of cytosolic *Salmonella* in HeLa epithelial cells through live cell imaging. It was observed that a population of cytosolic *Salmonella* was associated with p62 or LC3 and these bacteria replicated at a much faster rate compared to bacteria residing within the SCV, which showed little or no association with p62 or LC3 (Yu *et al.*, 2014). Furthermore, depletion of these autophagy components (p62 and LC3) significantly reduced replication of cytosolic *Salmonella* in HeLa cells (Yu *et al.*, 2014). In addition, a similar observation was reported in another study where a knockdown of Rab1, a GTPase required for autophagy of *Salmonella*, resulted in decreased *Salmonella* replication in HeLa epithelial cells (Huang *et al.*, 2011). These results have been suggested to indicate that autophagy facilitates *Salmonella* replication in epithelial cells.

According to a model proposed for p62-dependent autophagy favouring *Salmonella* replication in host cytosol, it is speculated that cytosolic *Salmonella* acquire nutrients which are supplied by autophagy for its replication (Yu *et al.*, 2014). This model further proposes that at least three populations of *Salmonella* exist within infected host cells. The first population is represented by bacteria which reside within the *Salmonella*-containing vacuole (SCV), where the bacteria appear to replicate slowly, as they do not associate with autophagosomes and have difficulty in acquiring nutrients from the cytosol. The second group represents those bacteria with damaged SCV membranes, and which are partially exposed to the host cell cytosol where they are inefficiently marked with ubiquitin and only partially associated with autophagosomes (Birmingham *et al.*, 2006). However, this population is able to acquire limited amount of nutrients and replicate faster. The third population of bacteria is completely exposed to cytosol and are efficiently marked with ubiquitin and show higher levels of association with autophagosomes. This third population acquires sufficient levels of nutrients and show the most rapid intracellular replication (Yu *et al.*, 2014). These hyper-

replicating bacteria lead to host cell death as a result of apoptosis at later time point's post-infection and are capable of initiating secondary infections.

The SCV is often regarded as a nutritionally-deprived compartment for the *Salmonella* as the pathogen has no or limited access to host cytosol (Dandekar *et al.*, 2012). Auxotrophic mutants of *Salmonella* have been reported to show defective intracellular replication and are also less virulent in a murine model of Typhoid (Fields *et al.*, 1986). Virulent *Salmonella* have been reported to modify the SCV in order to escape lysosomal killing and hence proliferate in host cells (Haraga *et al.*, 2008). The lysosomal killing of *Salmonella* within the SCV is by-passed as the SCV shows no or only very limited association with mannose-6-phosphate receptor (M6PO4), an essential marker necessary for lysosomal-mediated delivery of hydrolytic enzymes (Kornfeld and Mellman, 1989; Saftig and Klumperman, 2009). However, despite the nutrient limitation within the SCV, *Salmonella* is still able to replicate. This is indicative of a successful adaptation of *Salmonella* to this nutrient limited intracellular niche/environment. Our results provide the first evidence for a unique mechanism whereby intracellular *Salmonella* gain access to host cell cytosol from within its membrane-bound compartment to acquire nutrients.

The results of this study indicate that *Salmonella* Typhimurium acquires small peptides by co-opting the host cell chaperone-mediating autophagy (CMA) pathway of cytosolic protein turnover. CMA is a selective host cell protein turnover pathway involved in the transport of cytosolic proteins into lysosomes for degradation (Dice *et al.*, 2007; Orenstein and Cuervo, 2010). An estimated 30% of all cytosolic proteins are turned over through this mechanism (Wing *et al.*, 1991) and which is always active at basal levels in nearly all cell types (Kaushik and Cuervo, 2009). We have shown both in isolated intact SCV's and within host cells that the SCV is associated with the key components of CMA, LAMP-2A and Hsc73. Moreover, due to the ubiquitous nature of CMA in nearly all cell types, intracellular *Salmonella* would therefore have access to pre-formed amino acids even in non-absorptive

cell types such as macrophages. This would explain why *Salmonella* present within SCV successfully survives and replicates in infected macrophages whereas *Salmonella* present in the cytosol are killed in macrophages (Beuzón *et al.*, 2002).

Invasion assays using host cells deficient in LAMP-2A or inhibitors of CMA significantly affect the intracellular growth of the peptide-dependent mutant strain (KT7650) which can neither take up free, aromatic amino acids nor synthesize the aromatic amino acid phenylalanine. The results suggest that CMA as a source of amino acids contributes an estimated 20-30% to the intracellular growth of *Salmonella*, indicating that a significant fraction of the intracellular growth of *Salmonella* is independent of both *de novo* amino acids biosynthesis and free amino acids. However, fusion with lysosomes could serve as a source of peptides and amino acids, our results are consistent with a more restrictive interaction with lysosomal compartments, as indicated by the presence of both LAMP-1 and LAMP-2A on SCV's but exclusion of LAMP-2B, the mannose-6-phosphate receptor M6PO4, and LIMP-2, all markers which are present on mature lysosomal membranes but within particular micro-domains (Kaushik *et al.*, 2006).

As previously reported for cytosolic *Salmonella* using autophagy to acquire nutrients for replication in the cytoplasm, our results also suggest that *Salmonella* within the SCV can also acquire nutrients via a more specific form of autophagy, *i.e.* CMA, in order to support its intracellular growth within the SCV. By co-opting CMA, *Salmonella* has an additional advantage over cytosolic *Salmonella* in specialized cells such as macrophages. Both these observations imply a unique shift in the paradigm where host cellular pathways meant for elimination of intracellular pathogens are hijacked/utilized by *Salmonella* for its own benefit. This utilization of autophagy, either specific or general, by intracellular *Salmonella* also marks a unique adaptation towards host-pathogen interactions where a pathogen depending upon its intracellular state (either in cytosol or within SCV) adopts to that form of autophagy for its own survival.

Autophagy has recently been considered as an important defence mechanism by the host cells to clear intracellular microbes. However, it now appears that bacterial pathogens such as *Legionella*, *Coxiella*, *Yersinia*, *Brucella* etc. have evolved mechanisms to evade autophagic recognition or even co-opt the autophagy machinery for their own benefit as a replicative niche. Pathogens such as *Listeria* or *Shigella*, have evolved mechanisms to escape from their internalization vacuole into cytosol, and thus avoid phagolysosomal degradation (Ogawa *et al.*, 2005; Pay *et al.*, 2007). Some pathogens such as *Mycobacterium* (Stamm *et al.*, 2004) and *Legionella* (Joshi and Swanson, 2011) interfere with the conversion of this vacuole into a phagolysosomes. Other pathogens such as *Coxiella burnetii* and *Yersinia pseudotuberculosis* accumulate autophagy markers on the membrane of their vacuole and thus promote their own replication by autophagy (Romano *et al.*, 2010; Morea *et al.*, 2010).

Brucella abortus subverts the autophagy machinery to promote the infection process within host cells. Shortly after invasion of host cells, *Brucella* resides within the *Brucella* containing vacuoles (BCV). These vacuoles interact with endosomes and acquire endosomal markers and eventually fuse with ER-derived membranes and develop a replicative compartment (Celli *et al.*, 2004). These ER-derived BCV develop into autophagosome-like BCV (aBCV), a process similar to the formation of autophagosomes (Starr *et al.*, 2012). These observations from different intracellular pathogens strongly suggest that autophagy should not be strictly considered as an antibacterial host response to eliminate pathogens. Furthermore, these different means of avoiding or subverting autophagy highlight the degree of molecular complexity that underlies bacterial pathogen: host interactions, and point out difficulties in proposed therapeutic modulation of autophagy against bacterial infections.

Acquisition of CMA components

Infection of epithelial cells with intracellular pathogens such as *Shigella* and *Salmonella*, results in initiation of intracellular amino acid starvation conditions. This amino acid deprivation is induced due to damage in host cell membrane during the invasion process

(Tattoli *et al.*, 2012). However, in the case of *Salmonella* infections, this amino acid starvation stress condition within the host cell lasts only for a short period of time as the host cell membrane integrity is rapidly normalized. We suggest that it is this short-lived intracellular amino acid stress period induced during *Salmonella* infections results in the activation of various autophagy pathways, including CMA, and as a result during the course of SCV maturation, *Salmonella* acquires LAMP-2A. As the maturing SCV resembles an early endosome bearing markers such as EEA-1, vacuolar ATPases and other Rab proteins, the host cell may not distinguish between a maturing SCV and an early endosome, and therefore target LAMP-2A destined for endosomes to the SCV. As shown in figure xx , that SCV is associated with LAMP-2A as early as 1hr post-infection. Although LAMP-2A is acquired early during the infection process, it does not confirm that CMA is functionally active at this stage of infection.

The role of lipids and other host proteins have been extensively studied with regard to the membrane dynamics and integrity of the SCV. High levels of cholesterol are detected at the SCV membrane. Studies examining the importance of cholesterol during infections have reported that nearly 30-40% of total cellular cholesterol is recruited to the SCV (Catron *et al.*, 2002), where it plays an important role in T3SS translocon function (Hayward *et al.*, 2005). The *Salmonella* SPI-2 effector protein SseJ plays a role in esterification of cholesterol from the SCV membrane in infected cells. Furthermore, this esterification of cholesterol as lipid droplets by SseJ is critically important for bacterial intracellular replication (Nawabi *et al.*, 2008). Interestingly, CMA is activated upon removal of cholesterol from the lysosomal membrane either by chemical treatments, or under conditions of stress such as free radicals (Kaushik *et al.*, 2006). The reduction of cholesterol from lysosomal membrane allows LAMP-2A to multimerize, whereas it is normally present as a single molecule integrated in the membrane (Kaushik *et al.*, 2006). The rate limiting step in the CMA process is the multimerization step, which results in formation of the LAMP-2A translocon through which

target proteins are transported into the lysosomal lumen for degradation. Thus, we speculate that the esterification activity of SseJ on SCV membrane may result in reduction of the cholesterol levels in the SCV membrane and thereby allow previously acquired LAMP-2A to function, explaining why cholesterol esterification by SseJ is involved in promoting bacterial replication.

Implications of this study

Faecal shedding of *Salmonella* by asymptomatic carriers of *Salmonella* is often observed in humans and animals under stress conditions. One such example is increased faecal shedding of *Salmonella* Typhimurium in pigs and cattle upon transport (Isaacson et al., 1999; Callway et al., 2006). Reactivation of asymptomatic infections is therefore of high zoonotic importance, in particular for domestic animals in food production such as cattle and swine. Interestingly, CMA is activated under stress conditions (Orenstein and Cuervo, 2010), and the higher faecal loads may reflect a response of intracellular *Salmonella* to increased levels of nutrients due to the increased activity of CMA. In this manner, the intracellular growth of the pathogen is coupled to the fitness status of its host. Stress conditions experienced by the host would provide a nutritional shift within the SCV, resulting in increased proliferation of otherwise dormant *Salmonella*.

Outlook

During the course of this study we developed a novel method to successfully isolate intact *Salmonella*-containing vacuoles in macrophages using paramagnetic nanoparticles. The method described for isolation of SCV is rapid, gentle and could be utilised for analysing host proteins that interact with pathogen residing in a vacuole. These highly purified SCVs can be used for in-depth proteomic analysis of host proteins associated with the SCV in an effort to determine other possible pathogen: host interactions involved in intracellular survival and replication.

The identification of the chaperone-mediated autophagy pathway as not only interacting with the SCV, but also capable of delivering carbon-units and sources of amino acids, may also reveal a novel means of combatting intracellular pathogens. Host cell cytosolic proteins which are delivered to the lysosome for degradation through the CMA pathway require a KFERQ-targeting motif for recognition as a CMA substrate. This observation suggests new possibilities for the targeting of antibacterial peptides to this otherwise protected niche within host cells. Furthermore, it would be interesting to determine whether the other serovars of *Salmonella* and other intracellular pathogens residing in a vacuolar niche within the host cells can also access cytosolic peptides via the CMA pathway. The novel intracellular compartment markers and isolation methods and approaches resulting from this study may find far wider application in future studies in host: pathogen interactions.

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SUPPLEMENTS

Table 6. List of Cell Lines used in the study.

Cell Line	Description	Source or reference*
LoVo	Human colon intestinal epithelial cells	ATCC Cat. Nr. CCL-229 (Drewinko <i>et al.</i> , 1976)
THP-1	Human acute monocytic leukemia cells	DSMZ Cat. Nr. ACC-16 (Tsuchiya <i>et al.</i> , 1982)
HEK293T	Human kidney epithelial cells constitutively expressing the simian virus 40 (SV40) large T antigen	ATCC® CRL-11268
HEK293T/GAPDH-HT	HEK293T cells stably transfected with pCMV-GAPDH-HT plasmid under neomycin selection.	This study
HEK293T/KFERQ-DsRed	HEK293T cells stably transfected with pCDNA3.1-KFERQ-DsRed2 plasmid under neomycin selection.	This study
HEK293T/ LAMP-1 knockdown	HEK293T cells stably transfected with anti-LAMP-1 ShRNA plasmid to knockdown LAMP-1 under puromycin selection.	This study
HEK293T/ LAMP-2A knockdown	HEK293T cells stably transfected with anti-LAMP-1 shRNA plasmid to knockdown LAMP-2A under puromycin selection.	This study

Abbreviations: ATCC, American Type Culture Collection; DSMZ, Deutsche Sammlung von

Mikroorganismen und Zellkulturen

Table 7. List of Bacterial strains

Strain	Genotype/Relevant features	Source or reference
2994	SL1344 <i>S. Typhimurium hisG46 rpsL</i>	Hoiseth and Stocker, 1981
6220	$\Delta aroP \Delta pheP \Delta tyrP \Delta mtr \Delta pheA$	This study
6292	$\Delta aroP \Delta pheP \Delta tyrP \Delta mtr \Delta pheA \Delta trpBA$	This study
7586	$\Delta aroP \Delta pheP \Delta tyrP \Delta mtr \Delta trpBA$	This study
7598	$\Delta aroP \Delta pheP \Delta tyrP \Delta mtr \Delta tyrA \Delta tyrB$	This study
7740	$\Delta aroP \Delta pheP \Delta tyrP \Delta mtr \Delta tyrA \Delta trpBA$	This study
7650	$\Delta aroP \Delta pheP \Delta tyrP \Delta mtr \Delta pheA + GFP$	This study
7750	$\Delta aroP \Delta pheP \Delta tyrP \Delta mtr \Delta pheA(pACYC184)$	This study
7752	$\Delta aroP \Delta pheP \Delta tyrP \Delta mtr \Delta pheA (pMHEtetCDH)$	This study
7762	SL1344 (pACYC184)	This study
7764	SL1344(pMHEtetCDH)	This study
8010	$\Delta aroP \Delta pheP \Delta tyrP \Delta mtr \Delta livKHMGF$	This study
8016	$\Delta aroP \Delta pheP \Delta tyrP \Delta mtr \Delta pheA \Delta livKHMGF$	This study

Table 8. List of Plasmids

Plasmid	Relevant features	Reference/ source
pACYC184	<i>cat tet</i> p15a origin	This study
pMHEtetCDH	<i>cat tetOP-cdh⁺</i> p15a origin	Kleeb <i>et al.</i> , 2007
pcDNA3.1(+)	<i>bla neo</i> P _{CMV} SV40ori	Invitrogen
pCMV-GAPDH-HT	<i>bla neo</i> P _{CMV} SV40ori	Seki <i>et al.</i> , 2012

Table 9. List of primers

Name	Sequence (5'-3')	Gene	Reference
AROAH1P1	TCGGCGCGCCAGCCCGTCGACTGGCGCAACAG AAGACTTAGGCCATATGAATATCCTCCTTAG	<i>aroA</i>	This study
AROAH2P2	CTGTGGGGTTTTTATTTCTGTTTTTTGAGAGTT GAGTTTCATGTGTAGGCTGGAGCTGCTTCGA	<i>aroA</i>	This study
AROAF	TTTTCATCCCCACGGCCAGT	<i>aroA</i>	This study
AROAR	ATAAAAATAGTGCGAATACG	<i>aroA</i>	This study
AROPHF	CATGTTTCAAAGCTTTAACTGTGCACAAATGT	<i>aroP</i>	This study
AROPBR	CCGCTGATATGGATCCAATGGTTATACGTGGG	<i>aroP</i>	This study
PHEAH1P1	GGAGGCCTTTTTTATTGATAACAAAAAAGGCA ACACTATGGTGTAGGCTGGAGCTGCTTCGA	<i>pheA</i>	This study
PHEAH2P2	GTCGCAGGGATGAAGAAAAGCCGTTTTATTG AACGTCAGGCCATATGAATATCCTCCTTAG	<i>pheA</i>	This study
PHEAF	GAACAAGAAGGCCTCCCCCA	<i>pheA</i>	This study
PHEAR	CGCCATCTTCACCCTGCAGG	<i>pheA</i>	This study
MTRH1P1	AGATAGTCACACTATAAATGACTGGAGAGAG AGCTGAATGTGTAGGCTGGAGCTGCTTCGA	<i>mtr</i>	This study
MTRH2P2	GATGAAAACAGCTCATCCGGCTCGTTCAATTA CTGGTACACCATATGAATATCCTCCTTAG	<i>mtr</i>	This study
MTRF	CTGGTACAGTGCAGCGTATT	<i>mtr</i>	This study
MTRR	TACCATTTCGCGGACTTGTG	<i>mtr</i>	This study

PHEPH1P1	CTCAATAAAAAACGATACAGGGGTAGGACGTG AAAAACGCGGTGTAGGCTGGAGCTGCTTCGA	<i>pheP</i>	This study
PHEPH2P2	ATCGCATGAACATGGGATCGGGCAAAAAGT TAATGAGCCATATGAATATCCTCCTTAG	<i>pheP</i>	This study
PHEPF	CGGCAGCGAGAGCGAAATGT	<i>pheP</i>	This study
PHEPR	AGGGACATTGCGGCATTAAT	<i>pheP</i>	This study
LIVKH1P1	CAAAACATATAACATCACGAATGGGGATACA GGCACATGGTGTAGGCTGGAGCTGCTTCGA	<i>livK</i>	This study
LIVKF	GTCTGCTTTTAAACCAGCAA	<i>livK</i>	This study
LIVKR	TAAGGTAACCTTTTAAACC	<i>livK</i>	This study
LIVFH2P2	CACGCGCTTCCCCCTGGCACAGTCTAACCGC CCAGATAGCATATGAATATCCTCCTTAG	<i>livF</i>	This study
LIVFF	CGCCGGAAGAGATCCGCAAC	<i>livF</i>	This study
LIVFR	GAAAAATTTTACCGCTTGCC	<i>livF</i>	This study
TRPBAH1P1	CAGTTTTTCAGACACTGCGCGCATATTAAGGA AAAAACATGGTGTAGGCTGGAGCTGCTTCGA	<i>trpBA</i>	This study
TRPBAH2P2	CATTAAGCCGCCAGCGTTATGCTGACGGCTTA ACCCGTTACATATGAATATCCTCCTTAG	<i>trpBA</i>	This study
TRPBF	CAAAGATGCTCGTCTTCTGGC	<i>trpB</i>	This study
TRPBR	GGACAAAAGCGCCTTCCCGG	<i>trpB</i>	This study
TRPAF	CCGCGGAGATAAAGACATCT	<i>trpA</i>	This study

TRPAR	ATTCTGGTTCGGCGCCAGCC	<i>trpA</i>	This study
TYRAH1P1	GAGCGGCCAGCTGGCGGTGCGCGTCGCATAA GAGGTTGTTATGTGTAGGCTGGAGCTGCTTCG	<i>tyrA</i>	This study
TYRAH2P2	AGCCAGCAAAGCTGGCTTTTAGTATAGATGTC ATCATTATGGCATATGAATATCCTCCTTAG	<i>tyrA</i>	This study
TYRAF	CGTGAAATTCATAAAGATTT	<i>tyrA</i>	This study
TYRAR	CATAACGGTTCCTTAAACGA	<i>tyrA</i>	This study
TYRBH1P1	GTTATTTCTGCCCCTCTGTAAACCTGGAGAAC CATCGCGTGGTGTAGGCTGGAGCTGCTTCGA	<i>tyrB</i>	This study
TYRBH2P2	GGAGGGCACTGTTATCGCCGTCCGGCCTGAAT ATTACATGACCATATGAATATCCTCCTTAG	<i>tyrB</i>	This study
TYRBF	TCTCGTCCTTCCGGTTTATT	<i>tyrB</i>	This study
TYRBR	GATGATCACAACAGCTATTG	<i>tyrB</i>	This study
TYRPH1P1	CGGATATCACCGTTCTTTGTTACCGGGGTAGT AGAAAGCGTGTGTAGGCTGGAGCTGCTTCGA	<i>tyrP</i>	This study
TYRPH2P2	GTAATCTTTGCCTGGCGTTAACCTGGATCGGG CAGGAACCCCATATGAATATCCTCCTTAG	<i>tyrP</i>	This study
TYRPF	GTAATGCCTTACCCTGCGCC	<i>tyrP</i>	This study
TYRPR	CCATAGCGTGATGATTATCG	<i>tyrP</i>	This study

Table 10. Antibiotics used in the assays.

Antibiotic	Final concentration	Stock solution
Chloramphenicol	50µg/ml	10mg/ml
Gentamycin	50µg/ml & 10µg/ml	10mg/ml
Kanamycin	50µg/ml	10mg/ml
Neomycin	5µg/ml	1mg/ml
Puromycin	5µg/ml	1mg/ml
Tetracyclin	0.1µg/ml	100µg/ml

Table 11. List of primary Antibodies

Antibodies	Clone/Company	Type	Working conc.
Hsc 73	13D3 (Abcam)	Mouse mAb (IgM)	1/100 IF 1/5000 WB
LAMP-2A	(Abcam) Generated against a peptide corresponding to amino acids 350-410 of human LAMP-2A isoform	Rabbit pAb (IgG)	1/100 IF 1/5000 WB
LAMP-2B	Abcam	Rabbit pAb (IgG)	1/100 IF 1/5000 WB
LAMP-1	ID4B (Santacruz biotech)	Rat MAb (IgG2a)	1/100 IF 1/5000 WB
LIMP-2	C-18 (Santacruz biotech)	Goat pAb (IgG)	1/100 IF 1/5000 WB
M6PO4	Abcam	Goat pAb (IgG)	1/100 IF
GAPDH	Abcam	Rabbit pAb (IgG)	1/100 IF 1/5000 WB
Anti GFP	7G9(Acris)	Mouse mAb(IgG1)	1/5000

Note: - IF: - Immunofluorescence; WB: - Western blotting

Table 12. List of Secondary Conjugated Antibodies

Secondary Ab	Company/Conjugation	Used against	Working conc.
Goat-antimouse	Invitrogen/AF 546 Abcam /HRP	Hsc 73, anti-GFP	1/1000 IF 1/10,000 WB
Goat anti Rabbit	Invitrogen/AF 546 sara gifted/HRP	Lamp-2A, Lamp-2B GAPDH	1/1000 IF 1/10,000 WB
Goat anti Rat	Invitrogen/AF 546 Santacruzbiotech /HRP	LAMP-1	1/1000 IF 1/10,000 WB
Donkey anti Goat	Invitrogen/AF 546 Santacruzbiotech/HRP	LIMP-II	1/1000 IF 1/10,000 WB

Table 13. Solutions for SDS-Polyacrylamide gel

Reagents	5% (Stacking gel)	10% (Resolving gel)
Acrylamide (30%)	0.83 ml	3.3 ml
H₂O	3.4 ml	4.0 ml
1.5M Tris, pH 8.8	-	2.5 ml
1 M Tris, pH 6.8	0.63 ml	-
TEMED	0.005 ml	0.004 ml
APS (10%)	0.05 ml	0.1 ml
SDS (10%)	0.05 ml	0.1 ml

Table 14. Bacterial culture media

L-Broth (Lennox, 1955)	10 g/L Tryptone 5 g/L yeast extract 5 g/L NaCl pH 7
Lennox Agar (Lennox, 1955)	10 g/L Tryptone 5 g/L yeast extract 5 g/L NaCl 15 g/L Agar
M-9 glucose minimal media broth	10X M9 salts 20% Glucose 1M MgSO ₄ 1 mg/ml Ca ²⁺ -Pantothenate 1 mg/ml Thiamine 0,5M CaCl ₂
M-9 glucose minimal media agar	10X M9 salts 20% Glucose 1M MgSO ₄ 1 mg/ml Ca ²⁺ -Pantothenate 1 mg/ml Thiamine 0,5M CaCl ₂ 1.5% agar
DMEM/Ham's cell culture medium agar with 10% FCS	Dulbecco's modified Eagle's Medium (DMEM)/Ham's F-12 salts (1:1) (Biochrom, Berlin) 10% fetal calf serum (heat inactivated at 56°C for 30 min.) 15 g/L Agar

Protein studies

SDS-PAGE

APS-Stock solution	10% APS in H ₂ O
Coomassie-Staining solution	10% Acetic acid 45% Methanol 0.25% Coomassie Brilliant Blue R-250
Coomassie-Destaining solution	10% Acetic acid 20% Methanol
10X Running buffer	1.92 M Glycine 250 mM Tris-HCl 1% SDS
2 x Loading buffer	10% Glycerin 5% β -Mercaptoethanol 3% SDS 100 mM Tris-HCl 0.02% Bromphenolblue

Plasmid preparation buffers

E. coli

Solution I	25mM Tris-HCl pH 8.0 50mM Glucose
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Supplements

	2mM EDTA
Solution II	0.2 N NaOH 1% SDS
Solution III	58.9g Potassium acetate 50ml Glacial acetic acid 200ml ddH ₂ O
<i>Salmonella</i> (Kado)	
10X Kado TE	42.5g Tris base 1000ml ⁻¹ 7.5g EDTA 1000ml ⁻¹ in H ₂ O, pH 7.9, autoclaved
Kado Lysis Buffer	0.6g Tris base 100ml ⁻¹ 3g SDS 100ml ⁻¹ in H ₂ O, filter sterilized (0,45 µm)
Kado KOAc (Lösung III)	6ml of 5M KOAc 1.15ml of glacial HOAc 2.85ml ddH ₂ O

Laboratory instruments

Agarose gel electrophoresis tank	B1A and GE-B2, AGS, Heidelberg
Agilent 2100 Bioanalyzer	Agilent Technologies, UK
Aspirator	Vacuboy, neolab, heidelberg
Autoclave	GTA16, Gössner, Hamburg
Axon GenePix 4000AScanner	Axon Instruments, USA
Centrifuges	Rotina 46R, Hettich Zentrifugen, Tuttlingen
	3K30, Sigma Laborzentrifugen, Osterode
	5415D, eppendorf Zentrifugen, Engelsdorf
	Avanti J-25, Beckmann Coulter, Krefeld
Contrast microscope	Telaval 31, Zeiss, Jena
Nanodrop	peqLab, Erlangen
Electronic balance	BL3100, Sartorius, Göttingen
Electronic precision weighing instrument	BP210S, Sartorius, Göttingen
Electroporator	EasyjecT Prima, peqLab, Erlangen
Electrophoresis power supply	PS 250, Hybaid, Lexington, USA
Freezer -20 °C	Liebherr comfort GS1313, Ochsenhausen
Freezer -70 °C	Advantage, Nunc, Wiesbaden
Heating block	Thermomixer compact, Eppendorf, Hamburg
Hybridisation chambers	Anachem, UK
Image-Dokumentation instrument	UV-Transilluminator UVT 28 MP, Camera E.A.S.Y. 429 K, Documentationssystem RM6, Herolab, Wiesloch
Image-Dokumentation photo printer	P93E, Mitsubishi Electric, Ratingen
Incubators	CB150, Binder, Tuttlingen
	VT 5042 EK/N2, Heraeus, Hanau
LABMATE Precision pipettes	ABIMED GmbH, Langenfeld
Laser Scanning Confocal Microscope	Leica SP-II
Magnetic stirrer	IKAMAG RET, Th. Karow, Berlin
pH-Meter	Mikroprozessor pH-Meter 740, Knick, Berlin
Pipetting aid	Pipetus-akku, Hirschmann, Eberstadt
Refrigerator	Privileg 4873, Quelle, Fürth
Scanner	DUOSCAN T1200, AGFA, Köln
SDS-PAGE-apparatus	Biorad, USA
Shaking incubator	GFL3031, GFL, Burgwedel
Spectrophotometer	Ultrospec 3000 pro, Amersham, Freiburg
Sterile work bench	LaminAir HB 2448 and LB-48-C, Heraeus Hanau
Stratalinker	Stratagene, UK

Thermocycler	T3 Thermocycler, Biometra, Göttingen
Vacuum pump	N735 AN18, KNF Neuberger, Freiburg
Vortexer	MS2 Minishaker, IKA, Staufen
Waterbath	Grant OLS 200, CLF Laborgeräte, Burgwedel
	E-5, Julabo, Seelbach

Consumables

Amicon ultra centrifugal filters	Millipore, Germany
Cell culture flasks	Corning, Schiphol-Rijk, Netherland
Cell scraper	Corning, Schiphol-Rijk, Netherland
Nunc Cryotubes	Laborversand, Würzburg
Disposable cuvettes	MBT, Giessen
Disposable pipettes	Costar, Bodenheim
Electroporation cuvettes	2 mm, peqLab, Erlangen
Filter paper	Whatmanpaper 3MM, Schleicher and Schüll, Dassel
Pasteur pipettes	Roth, Karlsruhe
Petriplates	Sarstedt, Nürnberg
Sterile filters	Rotilabo-Spritzenfilter 0.45 µm and 0.22 µm pore size, Roth, Karlsruhe
12-well cell culture plates	CellBIND, Corning, Schiphol-Rijk, Netherland

Reagents

Acetic acid	Roth, Karlsruhe
Acrylamide-Bisacrylamide-Solution, Rotiphorese Gel 30,	RothKarlsruhe
Agar	Roth, Karlsruhe
Agarose	Roth, Karlsruhe
Alizarin yellow	Sigma-Aldrich, München
Ammoniumpersulfate (APS)	Sigma-Aldrich, München
Anhydrous 1,2-dichloroethane (DCE)	Sigma-Aldrich, UK
Anhydrous succinic anhydride	Sigma-Aldrich, UK
Antibiotics	Sigma-Aldrich, München
L-Arabinose	Roth, Karlsruhe
Boric acid	Roth, Karlsruhe
Bovine serum albumin (BSA)	Sigma-Aldrich, München
Bromophenol blue	Serva, Heidelberg
5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal)	Sigma-Aldrich, München
Calcium chloride	Roth, Karlsruhe
Chloroform	Merck, Darmstadt

Coomassie brilliant blue R250	Serva, Heidelberg
Cy3	GE Healthcare Lifesciences, UK
Cy5	GE Healthcare Lifesciences, UK
Diethylpyrocarbonate (DEPC)	Sigma-Aldrich, München
Dimethylsulfoxide (DMSO)	Roth, Karlsruhe
Dithiothreitol (DTT)	Sigma-Aldrich, München
DMEM/Ham's F-12 salts medium	Biochrom, Berlin
dNTPs	Roth, Karlsruhe
Ethanol pure	Merck, Darmstadt
Ethylenedinitrotetraacetic acid (EDTA)	Roth, Karlsruhe
Ethylene-glycol-bis-tetraacetic acid (EGTA)	Roth, Karlsruhe
Fetal calf serum	PAN Biotech, Germany
Gentamycin	Biochrom, Berlin
Glucose	Sigma-Aldrich, München
Glycerin	Roth, Karlsruhe
HPLC grade water	Roth, Karlsruhe
Iscoves modified DMEM	Biochrom, Berlin
Isopropyl- β -D-thiogalactopyranoside (IPTG)	Roth, Karlsruhe
Magnesium chloride	Roth, Karlsruhe
Magnesium sulphate	Roth, Karlsruhe
Methanol	Roth, Karlsruhe
Mowiol	Sigma-Aldrich, UK
n-Methylimidazole	Sigma-Aldrich, UK
o-Nitrophenyl- β -D-galactopyranosid (ONPG)	Sigma-Aldrich, München
PBS	Biochrom, Berlin
Phenol	Roth, Karlsruhe
Phenylmethylsulfonylfluoride (PMSF)	Roth, Karlsruhe
Potassium chloride	Roth, Karlsruhe
Random hexamers	Invitrogen, UK
Sodium carbonate	Merck, Darmstadt
Sodium chloride	Roth, Karlsruhe
Sodium citrate	Sigma-Aldrich, München
Sodium dihydrogen phosphate	Roth, Karlsruhe
di-Sodium hydrogen phosphate	Roth, Karlsruhe
Sodiumdodecyl sulfate(SDS)	Roth, Karlsruhe
TEMED	Biorad, USA
Tris Base	Sigma-Aldrich, München
Triton X-100	Sigma-Aldrich, München
Trypsin-EDTA	Biochrom, Berlin
Tryptone	Roth, Karlsruhe
Ultra-pure water	Sigma-Aldrich, UK
Wasserblau/Anilin blue	Fluka-Riedel-de Haën, Seelze
Xylene cyanol	Fluka-Riedel-de Haën, Seelze
Yeast extract	Roth, Karlsruhe

Commercial Kits

Abgene Extensor Hi-Fidelity PCR master mix	Thermo Scientific, UK
BCA Protein Assay Kit	Pierce, USA
BioPrime® DNA labeling system	Invitrogen, UK
100 bp-DNA Ladder	Roth, Karlsruhe
Lambda HindIII marker	Roth, Karlsruhe
Nanoparticles	TurboBeads, Zürich
Protein molecular weight marker VII-L	Sigma-Aldrich, München
Protino® Ni-TED column	Machery-Nagel, Düren
QIAGEN Plasmid Midi Kit	Qiagen, Hilden
QIAGEN Genomic DNA preparation kit	Qiagen, Hilden
Qia-quick PCR purification kit	Qiagen, UK

Enzymes

Bio Therm Taq-Polymerase	Rapidozym, Berlin
DNA-Restriction enzymes	Promega, Mannheim
Lysozyme	Roth, Karlsruhe
RNase free DNase	Promega, UK
T4 DNA-Ligase	Promrga, Mannheim

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Selbstständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Ich versichere, dass diese Arbeit in dieser oder anderer Form noch keiner anderen Prüfungsbehörde vorgelegt wurde. Der Inhalt der Promotionsordnung der Lebenswissenschaftlichen Fakultät I der Humboldt Universität zu Berlin vom 22 Dec, 2011 ist mir bekannt.

Berlin,

Vikash Singh

